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Factors influencing As and Sb bioavailability, accumulation and toxicity in agricultural plants

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Abstract

Various industrial activities can cause elevated concentrations of arsenic (As) and antimony (Sb) in soils, including near agricultural fields, leading to their accumulation in crops. This contamination may occur from individual metalloids or as co-contamination. Soil chemistry of the agricultural lands could be impacted by agricultural practices like addition of PO_4^{3-} containing fertilisers and waterlogging (which influences on chemical speciation due to anaerobic conditions). The aim of this thesis was to understand the influence of environmental and anthropogenic factors on the bioavailability of As and Sb in soils, and their accumulation and toxicity to agriculturally important plants under individual and co-contamination scenarios. This thesis focused on soil ageing, individual verses co-contamination of As and Sb, addition of PO_4^{3-} fertilisers and soil waterlogging affect.

In these experiments, bioavailability was determined by sequential extraction procedure (SEP) and compared to total soil concentration. Arsenic and Sb accumulation in plant tissues were assessed and compared with SEP-bioavailable fraction in the soils. All plants were grown in a controlled environment. Arsenic and Sb concentrations in soils and plant tissues were determined by ICP-MS. Plant toxicity of *Ipomoea aquatica* and *Brassica chinensis* were assessed using measurements of root and shoot dry mass, root and shoot length, and chlorophyll content. In *Oryza sativa*, the measurements of tissue dry mass and lengths, tiller and panicle number were used.

It is possible that As and Sb lability in soils change over the time, thus the effect of soil ageing on the bioavailability, accumulation and toxicity of As and Sb was assessed by comparing *I. aquatica* grown in historically (~34 years) and recently (spiked, 2 weeks) co-contaminated soils. *I. aquatica* seeds were germinated in contaminated soils and grown for 35 d within a controlled environment. Soil ageing decreased bioavailability of As and Sb in soil and accumulation in edible parts of *I. aquatica*. For shoot dry mass, As was more toxic in recently (EC_{50}: 11(4-30) mg/kg) than historically (EC_{50}: 49 (37-65) mg/kg) co-contaminated soil, with no difference observed for Sb between the soils ages. For shoot length, Sb was more toxic in recently (EC_{50}: 96 (42-219) mg/kg) compared to historically (EC_{50}: 12 (7-19) mg/kg) co-contaminated soils, with no difference observed for As. Aging is important in contaminated soils, it decreases the lability of As and Sb

and hence their bioavailability to agricultural plants, thus posing a lower risk of exposure of these metalloids to humans through agricultural plants grown in contaminated soils. Further investigations in this thesis were performed using spiked soils to reflect higher bioavailable concentrations of As and Sb in recently contaminated soils.

Antimony (Sb) is an emerging contaminant and until recently it was assumed to behave in a similar way to arsenic (As). Arsenic and Sb often co-occur in contaminated sites, yet most investigations consider their toxicity to plants singly. The interactive effects of As and Sb in recently contaminated soils (individually and co-contaminated) were investigated using *I. aquatica*. No differences in the bioavailability of As and Sb in soils were observed between individually and co-contaminated soils except at the highest Sb concentration of the co-contaminated soils. Exposure to individual Sb showed no visual toxicity on shoot dry mass and length while individual As showed an inhibition in both shoot dry mass and length. However, co-contaminated soil had a synergistic toxicity to shoot dry mass and additive toxicity to shoot length. These results showed that *I. aquatica* accumulates Sb to a higher level without showing obvious physical effects in the plant.

Application of PO_4^{3-} fertilisers to As contaminated soils may act as a competitive ligand for As and are known to increase the bioavailability and mobility of metalloids like arsenic (As). This may increase plant uptake of As and hence pose a risk to human health. Thus, the impacts of increasing PO_4^{3-} concentrations on bioavailability, accumulation and toxicity of As and Sb was investigated using *B. chinensis* grown in individually and co-contaminated soils. *B. chinensis* bioassays for low and high PO_4^{3-} were established for 40 d within a controlled environment. The increased soil PO_4^{3-} (500 mg P/kg) in individually contaminated had no impact on the bioavailability of As and Sb in the soil. In co-contaminated soil, PO_4^{3-} decreased As bioavailability at high As exposures whereas Sb bioavailability was not affected by PO_4^{3-} except when Sb in soil ≥ 1680 mg/kg. In individually contaminated soil, the accumulation of As and Sb increased at high PO_4^{3-}, whereas in co-contaminated soil, As and Sb accumulation increased only within the low soil concentrations. Arsenic was shown to be toxic for both shoot and root dry mass at low PO_4^{3-} soil. In contrast, no toxicity was observed for As at high PO_4^{3-} soil. In regards to individual verses co-contamination, shoot dry mass suggested that As in the $As_{(Individual)}$ (EC_{50}: 42 (21-63) mg/kg) was more toxic than As in the $As + Sb_{(Combined)}$ (EC_{50}: 72 (53-91) mg/kg) whereas no significant difference was observed in the root dry mass.

Antimony in individual and co-contaminated soil showed no toxicity at either low or high PO_4^{3-} soils. The addition of high soil PO_4^{3-} concentrations in contaminated soils may ameliorate As toxicity despite the accumulation of high As concentrations in plants tissues.

Environmental conditions, such as soil flooding or waterlogging, greatly influence the speciation of As and Sb due to changes in soil redox potential resulting from fluctuations in oxygen and other electron acceptors. These fluctuations can change the oxidation states of As and Sb which alters their mobility, uptake and toxicity to plants. Waterlogging is common in paddy soil and thus, its impacts on bioavailability, accumulation and toxicity of As and Sb was assessed using *O. sativa* grown in individually and co-contaminated soils. Germinated *O. sativa* seeds were transferred to pots at the three leaf stage seedlings and placed in a controlled environment at 20°C (night) and 28°C (day). Results showed no observed difference in the bioavailability of As and Sb in individually contaminated compared to co-contaminated soils. Accumulation of As and Sb in *O. sativa* decreased in the order of root > shoot > grain and was not affected by co-contamination. The co-occurrence of As and Sb had an additive toxicity on tiller number, shoot and root dry mass; synergistic toxicity on shoot and root lengths; and antagonistic toxicity on panicle number and grain dry mass.

This thesis showed, As and Sb bioavailability decreased with soil ageing and addition of high PO_4^{3-} concentrations (only at high soil As and Sb concentrations) while no significant difference was observed within co-contaminated soils compared to their individually contaminated soil at both aerobic and waterlogged conditions. Arsenic and Sb accumulation in edible parts of tested agricultural plants decreased with soil ageing, however, accumulation was increased in the presence of high PO_4^{3-} concentrations in individually contaminated soils. Interestingly, in co-contaminated soil As and Sb accumulation increased only within the low soil concentration. Similar to the bioavailability, there was no change observed in the accumulation of As and Sb in *I. aquatica* under co-contamination and to *O. sativa* under waterlogged conditions. Furthermore, co-occurrence of As and Sb showed additive and synergistic interactions in *I. aquatica* under co-contamination and to *O. sativa* under waterlogged conditions. In summary, As accumulation and toxicity to all tested agricultural plants grown under different soil conditions was much higher than that of Sb. Antimony was not toxic to *I.*

aquatica and *B. chinensis*, but toxic effects were observed on shoot and root dry mass of *O. sativa.* Phosphate in soil increased As and Sb accumulation, however, no toxic effects were visually observed increasing the risk As and Sb may pose to human health through dietary exposure. This thesis has important implications for determining the quality of crops grown in co-contaminated soils and potential risks of agriculturally important species to human health and provides new information to guide agricultural practices including risk assessment of As and Sb contamination.

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Certification

I, Lakmini Pramodya Egodawatta, declare that this thesis submitted in fulfilment of the requirements for the conferral of the degree of Doctor of Philosophy in the School of Earth, Atmospheric and Life Sciences, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

Lakmini Pramodya Egodawatta

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Abbreviations

ADP- Adenosine di-phosphate

ATC- Automatic temperature compensation

BAF- Bioaccumulation factor

CI- Confidence interval

CRM- Certified reference materials

DMA- Dimethylarsine

EC- Effective concentration

EDI- Effective daily intake

FA- Fulvic acids

GSH- Glutathione

GSSG- Glutathione disulphide

HS- Historically contaminated soil

ICP-MS- Inductively coupled plasma-mass spectrometer

LOI- Loss on ignition

MMA- Monomethylarsine

NOEC- No observed effect concentration

PAM- Pulse-amplitude modulated

PC- Phytochelatin

PHT- Phosphate transporters

PTFE- Polytetrafluoroethylene

ROS- Reactive oxygen species

RS- Recently contaminated soil

SEP- Sequential extraction procedure

SOM- Soil organic matter

TDI- Tolerable daily intake

TF- Translocation factor

TKN- Total kjeldhal nitrogen

TMA- Trimethylarsine

TMAO- Trimethylarsine oxide

TOC- Total organic carbon

Thesis Publications

1. **Egodawatta, L.P**., Holland, A., Koppel, D., Jolley, D.F. Assessment of As and Sb bioavailability and toxicity to rice (*Oryza sativa* l.) cultivated in individually and co-contaminated soils, under waterlogged conditions. Environmental Toxicology and Chemistry. *manuscript in preparation*. (Chapter 6).
2. **Egodawatta, L.P.**, Holland, A., Koppel, D., Jolley, D.F. 2020. Influence of soil phosphate on accumulation and toxicity of As and Sb in choy sum cultivated in individually and co-contaminated soils. *Environmental Toxicology and Chemistry*. DOI: 10.1002/etc.4708 (Chapter 5).
3. **Egodawatta, L.P.**, Holland, A., Koppel, D., Jolley, D.F. 2020. Interactive effects of arsenic and antimony on Ipomoea aquatica growth and bioaccumulation in co-contaminated soil. *Environmental Pollution*. 259, 113830. (Chapter 4).
4. **Egodawatta, L.P.**, Macoustra, G.K., Ngo, L.K., Jolley, D.F. 2018. As and Sb are more labile and toxic to water spinach (Ipomoea aquatica) in recently contaminated soils than historically co-contaminated soils. *Environmental Science: Processes & Impacts*. 20(5), 833-44. (Chapter 3).

Thesis Presentations

- Egodawatta, L.P., Holland, A., Koppel, D. and Jolley, D.F. *Influence of phosphate containing fertilisers on the growth of choy sum and uptake of As and Sb in contaminated soils*, Oral session presented at SETAC Australasia, Darwin, Australia; July 2019.

- Egodawatta, L.P., Holland, A. and Jolley, D.F. *Bioavailability of Arsenic and Antimony co-contamination to vegetable crops grown in agricultural soils*, Oral presentation at University of Orléans, France; May 2018.

- Egodawatta, L.P., Holland, A. and Jolley, D.F. *Bioavailability of Arsenic and Antimony co-contamination to vegetable crops in agricultural soils*, Oral session presented at SETAC Europe, Rome, Italy; May 2018.

- Egodawatta, L.P., Jolley, D.F., and Bennett, W.W. *Impacts of arsenic and antimony co-contamination on leafy vegetables grown in contaminated soils*, Oral session presented at SETAC Australasia, Gold Coast, Australia; September 2017.

- Egodawatta, L.P., Macoustra, G., Ngo L.K., and Jolley, D.F. *Arsenic and antimony lability in recently and historically contaminated soils and its effects on water spinach.* Poster session presented at Biogeochemical cycles of metalloids (As, Sb, Hg and Se) in the modern and ancient, Goldschmidt conference, Yokohama, Japan; June 2016.

- Egodawatta, L.P., Macoustra, G., Ngo L.K., and Jolley, D.F. *Arsenic and antimony lability in recently and historically contaminated soils and its effects on water spinach*. Oral session presented at 23rd Annual RACI R&D Topics Analytical and Environmental Chemistry Conference, Melbourne, Australia; December 2015.

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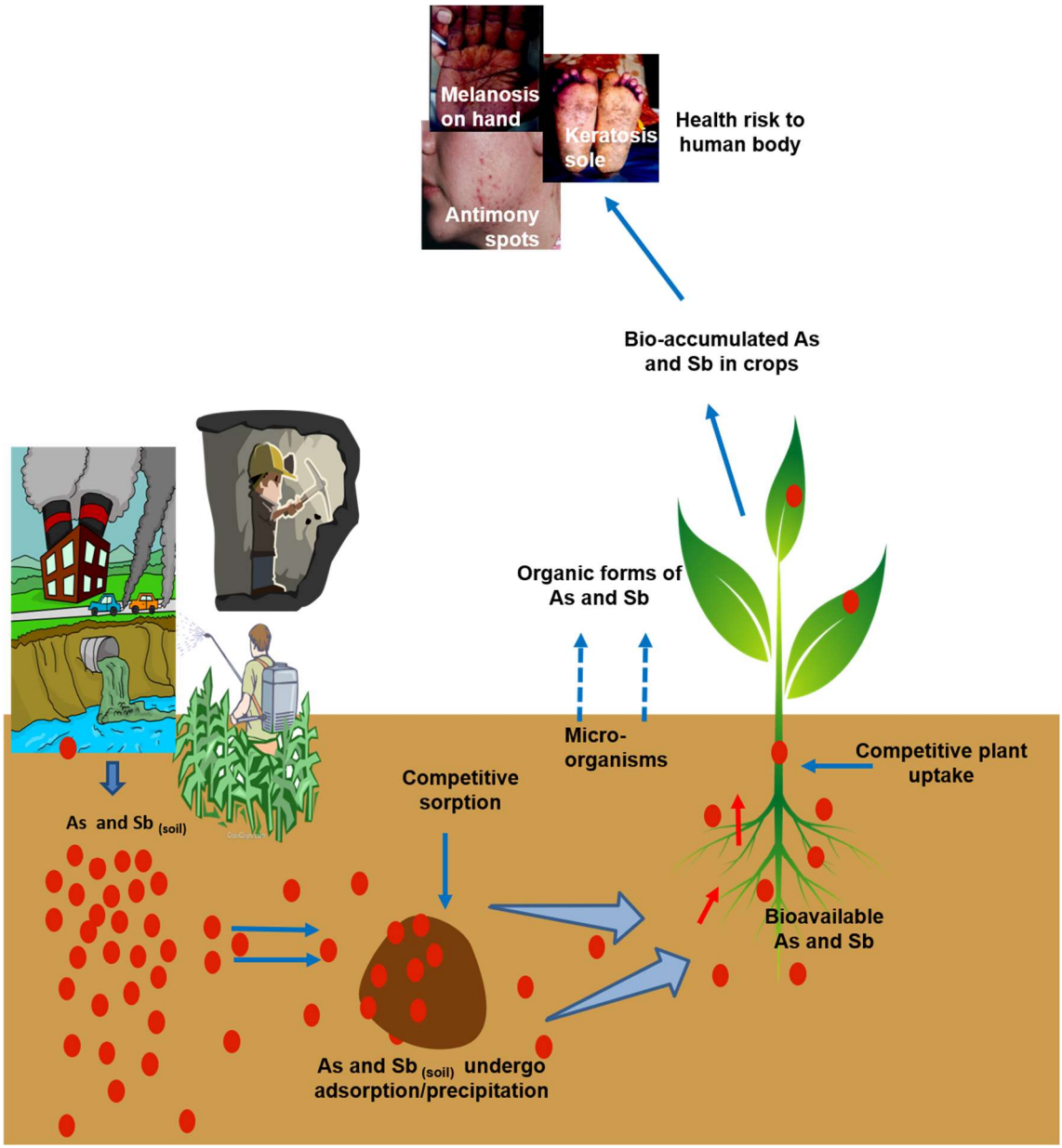
Chapter 1. Introduction



1.1 Background

Arsenic (As) naturally co-occurs with antimony (Sb) as trace elements in the geosphere. They accumulate in various environmental compartments (such as soil, ground and surface water, air and microbial, plant and animal communities) through various emission sources causing contamination of the receiving environment (Sadiq 1997, Komarek et al. 2013). Both As and Sb are metalloids and are in the same group of the periodic table (Group 15). They exhibit the same range of oxidation states (-III to V) and occur frequently as tri(III)- and penta(V)- valent oxyanions in soils and aqueous environments (Sadiq 1997, Wilson et al. 2010, Okkenhaug et al. 2012, Abbas et al. 2018) and are believed to behave chemically similar in the environment.

Arsenic and Sb have no known beneficial biological functions and are toxic at elevated concentrations (Casado et al. 2007). Unlike organic contaminants, most metals do not undergo microbial or chemical degradation and thus pose a long-lasting risk to the environment and human health through possible exposure via the food chain and drinking water (Wuana et al. 2011, Bolan et al. 2014). Exposure to elevated concentrations of As and Sb has been shown to cause liver, skin, lung, bladder and kidney cancers and cardiovascular diseases in humans (Gebel et al. 1998, Wu et al. 2011). As a result, both As and Sb have been recognized as global pollutants by health and environment regulation bodies, including in the United States of America and European Union (Wu et al. 2011, Alvarez-Ayuso et al. 2013, Fu et al. 2016).

Soils globally have been contaminated with As and Sb, from activities such as mining or smelting, or from naturally occurring minerals (Flynn et al. 2003, Telford et al. 2009, Barać et al. 2015, Fu et al. 2016, Abad-Valle et al. 2018). The risk posed by these metalloids to the environment strongly depends on their bioavailability in soils. Bioavailability determines the proportion of metalloids of the total that is readily mobilize and available for plant uptake. This fraction is controlled by many chemical processes such as adsorption-desorption reactions, chemical complexation with organic/inorganic ligands, biotic/abiotic redox reactions and precipitation-dissolution reactions. In particular, the bioavailability of As and Sb in soils depends on the surrounding soil chemistry, the metalloids' chemical speciation and time since contamination.

Arsenic and Sb have both been shown to be toxic to plants and accumulate in plant tissues

(Finnegan et al. 2012, Feng et al. 2013, Abbas et al. 2018). This accumulation and toxicity depends on their biogeochemical behaviour (such as speciation and bioavailability) in the soil and the species of plant being exposed (Sadiq 1997, Gulz et al. 2005, Casado et al. 2007, Zeng et al. 2015). The accumulation of As and Sb in agricultural plants is of concern because of their contribution to the diet of humans and animals. Therefore, contaminated agricultural plants may pose a risk to animal and human health (Wu et al. 2011, Pierart et al. 2015, Ngo et al. 2016). However, factors contributing to this risk are not fully understood yet.

1.2 Arsenic and Sb in soils

1.2.1 Occurrence and distribution

Arsenic and Sb are common metalloids naturally occurring in rocks and soils. Their background concentrations may be affected by the parent rock material, local climate conditions, organic and inorganic components, and redox potential of the soil (Herath et al. 2016). The As and Sb concentrations in uncontaminated soils usually range from 1-40 mg/kg (Herath et al. 2016) and 0.3-8.6 mg/kg, respectively (Tschan et al. 2009).

Arsenic is the 20th most abundant element in the earth's crust with average crustal concentrations of 1.8 mg/kg (Bissen et al. 2003, Herath et al. 2016). Even though As occurs in a variety of natural reservoirs, such as oceans, the atmosphere, biota, rocks and soils, more than 99% of As is associated with rocks and minerals (Herath et al. 2016). For example, As is the major constitute of ~245 mineral species, 60% as arsenates, 20% as sulphides and sulphosalts, and the remaining 20% as arsenides, arsenites, oxides, silicates, and elemental As (National Research Council Committee on et al. 1977, Herath et al. 2016). The most important As minerals, mainly associated with sulphur and oxides, are shown in Table 1.1(Bissen et al. 2003, Wilson et al. 2010, Herath et al. 2016). Arsenic can also exist in organic forms, typically methylated species, which can be converted into CO_2 and inorganic As by oxidative degradation or into volatile As compounds like arsine (AsH_3) gas by reduction (Arai 2010).

Natural concentrations of Sb in the environment are lower than As with average crustal concentrations of 0.2-0.3 mg/kg (Tschan et al. 2009). Antimony forms over 100 different minerals with S, Pb, O and As (some of the primary minerals are shown in Table 1.1) (Arai 2010, Wilson et al. 2010, Herath et al. 2017). Additionally, organic Sb can be

methylated and volatilized, forming compounds like SbH_3, or remain as a methylated species in the environment (Arai 2010).

1.2.2 Sources of contamination

In recent years, there has been an increase in As and Sb concentrations in surface soil and water due to natural and anthropogenic activities. Natural sources of As and Sb enrichment are mainly associated with unique geological conditions that promote their mobilisation or leaching from minerals (Camacho et al. 2011). For example, the weathering of primary minerals has been identified as the major natural source of As enrichment in soil. This is reported to contribute an annual global input of 45,000 Mg As/year to soils (Arai 2010). This process has led to high As and Sb concentrations in the groundwater and soils of many countries (Herath et al. 2016, Wen et al. 2016); including, Bangladesh, India, China, Taiwan, Vietnam, USA, Argentina, Chile, Mexico, Australia, New Zealand, Japan, Spain and Slovakia (Arai 2010, Fu et al. 2016, Herath et al. 2016, Abbas et al. 2018). Other natural sources of As and Sb enrichment in soils include atmospheric deposition, volcanic eruptions, geothermal/hydrothermal activity and forest fires (Bissen et al. 2003, Garelick et al. 2009). The concentrations of As and Sb associated with soils of different parent minerals, including igneous and sedimentary rocks, are given in Table 1.2.

Anthropogenic activities that lead to As contamination in the environment are primarily associated with industrial processes which include the smelting of As bearing minerals, mining activities and fossil fuel combustion (Bissen et al. 2003, Arai 2010). For example, Bissen et al. (2003) estimated that 62,000 t of As is emitted annually from industry with ~80% by copper smelters. Arsenic is also used in pharmaceutical and glass industries, in the manufacturing of alloys, sheep dips, wood and leather preservatives, arsenic-containing pigments, antifouling paints, and biocides and poison baits (Chung et al. 2014). Arsenic contamination of agricultural lands has typically resulted from the use of arsenical pesticides, fungicides (such as $PbHAsO_4$) or wood preservatives (such as chromated copper arsenate) (Hingston et al. 2001, Bissen et al. 2003, Arai 2010).

Table 1.1. Common As and Sb species found in soils and aqueous environments. Modified from Wilson et al. (2010).

	As		Sb	
	Name	**Formula**	**Name**	**Formula**
Minerals	Arsenopyrite	FeAsS	Stibnite	Sb_2S_3
	Orpiment	As_2S_3	Valentinite	Sb_2O_3
	Realgar	AsS	Senarmontite	Sb_2O_3
	Arsenolite	As_2O_3	Cervantite	Sb_2O_4
	Olivenite	Cu_2OHAsO_4	Kermisite	Sb_2S_2O
	Proustite	Ag_3AsS_3	Guettardite	$Pb(Sb, As)_2S_4$
	Niccolite	NiAs	Annivite	$Cu_{12}(As, Bi, Sb)_4S_{13}$
	Cobaltite	CoAsS	Gabrielite	$Tl_6Ag_3Cu_6(As, Sb)_9S_{21}$
	Tennantite	$Cu_{12}As_4S_{13}$	Stibio-domeykite	$Cu_3(As, Sb)$
	Enargite	Cu_3AsS_4	Geocronite	$Pb_5(As, Sb)_{12}S_8$
Aqueous species (+5 oxidation state)	Arsenic acid	$AsO(OH)_3$ or H_3AsO_4	Antimonic acid	$Sb(OH)_5$
	Dihydrogen arsenate	$AsO_2(OH)_2^-$ or $H_2AsO_4^-$	Antimonate	$Sb(OH)_6^-$ (or SbO_3^-)
	Monohydrogen arsenate	AsO_3OH^{2-} or $HAsO_4^{2-}$		
Aqueous species (+3 oxidation state)	Arsenous acid	$As(OH)_3$ or H_3AsO_3	Antimonous acid	$Sb(OH)_3$ (or $HSbO_2$)
	Arsenite	$AsO(OH)_2^-$ or $H_2AsO_3^-$ or $HAsO_3^{2-}$	Antimonite	$Sb(OH)_2^+$
			Sulphidic complexes	$H_2Sb_2S_4$
				$HSb_2S_4^-$
				$Sb_2S_4^{2-}$
Methylated species	Monomethylarsonic (MMA)	$CH_3AsO(OH)_2$	Methylstibonic acid (MSA)	$(CH_3)SbO(OH)_2$
	Dimethylarsinic (DMA)	$(CH_3)_2As(O)OH$	Dimethylstibonic acid (DMSA)	$(CH_3)_2Sb(O)OH$
	Dimethylarsine	$(CH_3)_3As$	Trimethylstiboxide	$(CH_3)_3Sb$

Most Sb contamination in soil originates from anthropogenic activities including mining, smelting and leaching from bullets at shooting ranges (Arai 2010). In contrast to As, Sb has low abundance within the Earth's crust. Yet, in recent decades the global annual production of Sb has been ~4 fold greater than that of As (Fu et al. 2016). Antimony is used in specific manufacturing and chemical applications, with 52% of produced Sb used in the production of flame retardants, 38% used in lead alloys and lead-acid batteries, 6% used as catalysts to produce the common plastic polyethylene terephthalate and less than 3% used in other chemicals and ceramics (Fu et al. 2011, He et al. 2019).

Previous studies have shown that both As and Sb co-occur in the environment and are found together in extremely high concentrations in soils adjacent to mining and smelting sites (He 2007, Li et al. 2014, Ngo et al. 2016, Doherty et al. 2017). For example, the average As and Sb concentrations in Chinese Xikuangshan (XKS) mine soils have been reported to range between 14-256 mg/kg and 144-3948 mg/kg, respectively (Zeng et al. 2015). Arsenic and Sb concentrations at a mine site in Slovakia were also to be as high as 5166 mg/kg and 9861 mg/kg, respectively (Hiller et al. 2012). These contaminated soils may lead to As and Sb transportation to nearby agricultural lands through atmospheric deposition and contaminated irrigation water, causing further agricultural soil contamination. Some studies have reported that As and Sb concentrations in agricultural land near a mine dump range between 138-379 mg/kg (Abad-Valle et al. 2018) and 3.12-131 mg/kg (He et al. 2019), respectively. Further, paddy soils near XKS mine sites were also contaminated with As and Sb with an average concentration of 78 mg/kg and 1562 mg/kg, respectively (Okkenhaug et al. 2012). While As and Sb often co-occur in contaminated sites, very little environmental research has been conducted on them as mixtures, their interactive effects and toxicity.

Table 1.2. Lithogenic sources of As and Sb. Units are in mg/kg. Modified from Alloway (2013).

Element	Granite granodiorite	Gabbo salt	Ultramafic rocks	Sand stone	Shales	Black/oil shales	Limestones	Coal
As	3	0.7	0.7	0.5	13	<500	1.5	10
Sb	0.3	0.2	0.1	0.05	1	<10	0.15	2

1.2.3 Chemistry

The occurrence of an element in different chemical forms, oxidation states, and mineral phases is known as its chemical speciation (Abbas et al. 2018). Environmental factors such as soil pH and redox state are the primary factors which control the transformation and distribution of chemical speciation in the natural environment (Wilkie et al. 1996, Sadiq 1997, Wilson et al. 2010, Abbas et al. 2018). For example, As and Sb may be present in a variety of species, such as free ionic forms, precipitated as solids, adsorbed onto soil organic and/or inorganic constituents, and/or exchangeable or structural constituents of primary and secondary minerals (Abbas et al. 2018). These species have different abilities to form bonds with soil compartments, influencing their mobility in the soil, which will affect their bioavailability and toxicity (Bostick et al. 2003, Wilson et al. 2010).

The redox potential (Eh) of surface soils varies from ~+500 mv (strongly oxidising conditions) to ~(-)300 mv (strongly reducing conditions) (Signes-Pastor et al. 2007). Fluctuations in redox conditions can affect the speciation of As and Sb in soil systems (Sadiq 1997). Eh–pH diagrams illustrate the interactions between redox and pH at a state of equilibrium (see Figure 1.1 for As). Under aerobic conditions typical of surface soils (Eh > 400 mV) and low pH (less than ~ 6.9), the predominant As(V) species is $H_2AsO_4^-$ (pK_1=2.20), whereas at higher pH, $HAsO_4^{2-}$ (pK_2=6.97) is predominant. H_3AsO_4 and AsO_4^{3-} (pK_3=13.4) are present only in extremely acidic and alkaline conditions, respectively (Arai 2010). Under anaerobic conditions typical of waterlogged soils (Eh < -100 mV) and with a pH less than 9.2, the dominant species As(III) is present in the form of H_3AsO_3 (pK_1=9.22 and pK_1=12.13) (Sadiq 1997, Arai 2010, Wilson et al. 2010, Rouwane et al. 2016). $H_2AsO_3^-$ is present only at extremely alkaline conditions.

The redox behaviour of Sb is shown in Eh–pH diagram of Sb presented in Figure 1.2. Under aerobic conditions, $Sb(OH)_6^-$ is the most stable form of Sb(V) over a wide pH

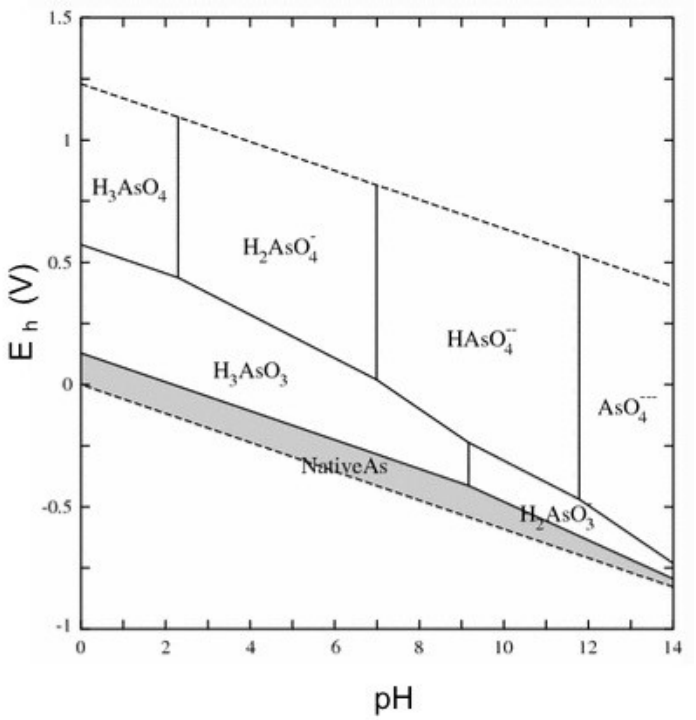


Figure 1.1. Eh–pH diagram for the system As–O–H at 25°C and 1 bar total pressure. Dissolved arsenic concentration of 10^{-6} mol/L (Gray area denotes solid phase). Figure taken from Lu et al. (2011).

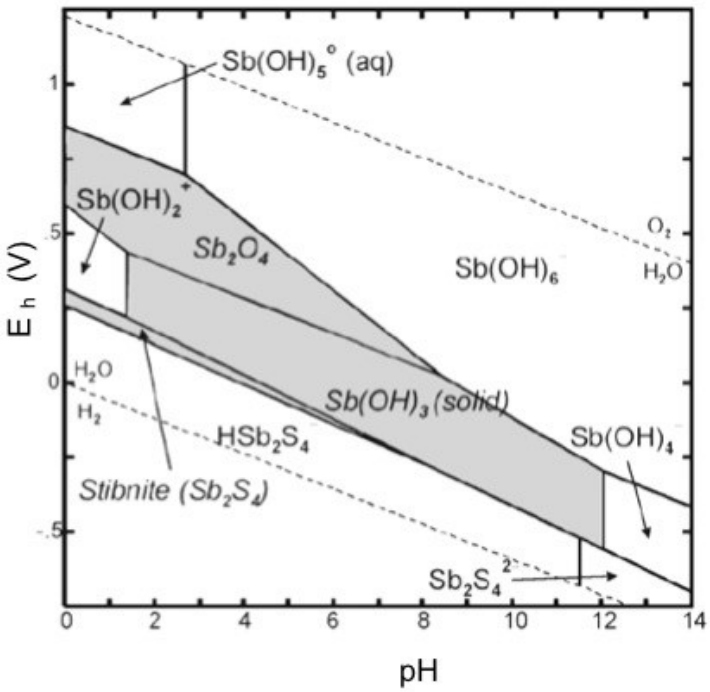


Figure 1.2. Eh–pH diagram of antimony in the Sb–H_2O system at 25°C and 1 bar total pressure. Dissolved antimony concentration of 10^{-7} mol/L (Gray area denotes solid phase) and a dissolved sulphur concentration of 10^{-3} mol/L. Figure taken from Krupka et al. (2002).

range, whereas under anaerobic conditions, Sb(III) is predominantly present in the form of neutral $Sb(OH)_3$ in the pH range of 2 to 11. Antimony will readily form sulphur complexes under reducing conditions, such as $Sb_2S_{3(s)}$ in low to intermediate pH conditions and SbS_2^- under high pH conditions (Filella et al. 2002).

1.2.4 Molecular configurations

Arsenic and Sb are in the same group of the periodic table (Group 15) and have similar s^2p^3 outer orbital electronic configurations. Thus, they exhibit the same range of oxidation states (-III to V) and occur frequently as tri(III)- and penta(V)- valent oxyanions in soils and aqueous environments (Sadiq 1997, Wilson et al. 2010, Okkenhaug et al. 2012, Abbas et al. 2018). In natural aerobic soils, both As and Sb exist in their +V oxidation state as arsenate (AsO_4^{3-}) and antimonate (SbO_4^{3-}) whereas in anaerobic soils such as flooded rice-paddies, they are present in their +III oxidation state as arsenite (AsO_3^{3-}) and antimonite (SbO_3^{3-}). When considering the geometric configurations of their +V state, AsO_4^{3-} is tetrahedral whereas $Sb(OH)_6^-$ is octahedral (Wilson et al. 2010). Both oxyanions in their +III state, however, form trigonal pyramidal molecular geometries. Although As and Sb are assumed to be similar in soil and aqueous environments due to their similar oxidation states, a few studies have shown that As and Sb have contrasting geochemical behaviours (Mitsunobu et al. 2006, Fu et al. 2016). The chemical similarities and differences of As and Sb may influence their relative bioavailability, bioaccumulation and toxicity when they co-occur in soils, such as those surrounding mine sites (see Section 1.2.2).

1.3 Mobility and bioavailability of As and Sb in soils

Generally, metalloid uptake in plants increases with increasing soil concentration, but this is dependent on the mobility and bioavailability of the metalloids in the soil (Intawongse et al. 2006, Ngo et al. 2016). The fraction of elements which can be readily mobilized in the soil environment and taken up by plants is known as the "bioavailable fraction" (Intawongse et al. 2006). The bioavailable fraction of metalloids in soils is often influenced by adsorption-desorption reactions, chemical complexation with organic and inorganic ligands, biotic and abiotic redox reactions and precipitation-dissolution reactions (Figure 1.3) (Pigna et al. 2010). In soil solutions, As and Sb may undergo processes including surface precipitation/surface oxidation, Ostwald ripening, cavity entrapment, diffusion into microspores and/or incorporation into crystal lattices of the

soils (Naidu et al. 2008, Violante et al. 2008, Wang et al. 2015).

1.3.1 Adsorption-desorption mechanisms

Adsorption-desorption reactions are the main processes which control the mobility of As and Sb in soils (Wilson et al. 2010). The soil sorbents responsible for the adsorption of As and Sb oxyanions are phyllosilicates, soil organic matter (SOM), metal oxides (crystalline and amorphous iron (Fe), aluminium (Al) and manganese (Mn) (hydro)oxides), carbonates and organo-mineral complexes (Pigna et al. 2015, Caporale et al. 2016). However, the extent of adsorption or desorption can be influenced by factors such as soil pH, type of sorbent (sorption capacities, cation and anion exchange capacities and binding energies), concentrations of competitive (organic and inorganic) ligands and redox state of the anions (Violante et al. 2010, Wilson et al. 2010, Pigna et al. 2015). In environmental conditions, the adsorption of As and Sb oxyanions to variable-charged mineral surfaces occurs via inner and outer sphere complexation (Arai et al. 2001, Caporale et al. 2016). Inner-sphere surface complexes form covalent linkages with no water molecule between the adsorbed ion and the surface functional group (Manning et al. 1998, Goldberg et al. 2001). Outer-sphere surface complexes form electrostatic interactions with at least one water molecule (Caporale et al. 2016). Thus, outer-sphere surface complexes are considered as weaker interactions compared to inner-sphere surface complexes (Figure 1.4).

Arsenic and Sb species may also form different surface complexes through different linkages such as monodentate, bidentate-binuclear, and bidentate-mononuclear complexes depending on the pH, ionic strength and surface area (or sorption site density) (Violante 2013, Caporale et al. 2016).

Oxides and hydroxides

Oxides and hydroxides, such as Fe, Al and Mn (hydr)oxides, are formed by the weathering of primary minerals and are some of the main components responsible for As and Sb adsorption within the natural environment (Huat et al. 2012). However, they have been shown to have different adsorption capacities for different As and Sb species depending on their surface charge and the metalloid's speciation which are highly pH dependent. For example, at a high pH, Fe oxides surfaces have negative charges whereas at low pH, they tend to have more of a positive charge on the surface offering more sites for anionic adsorption (Sadiq 1997).

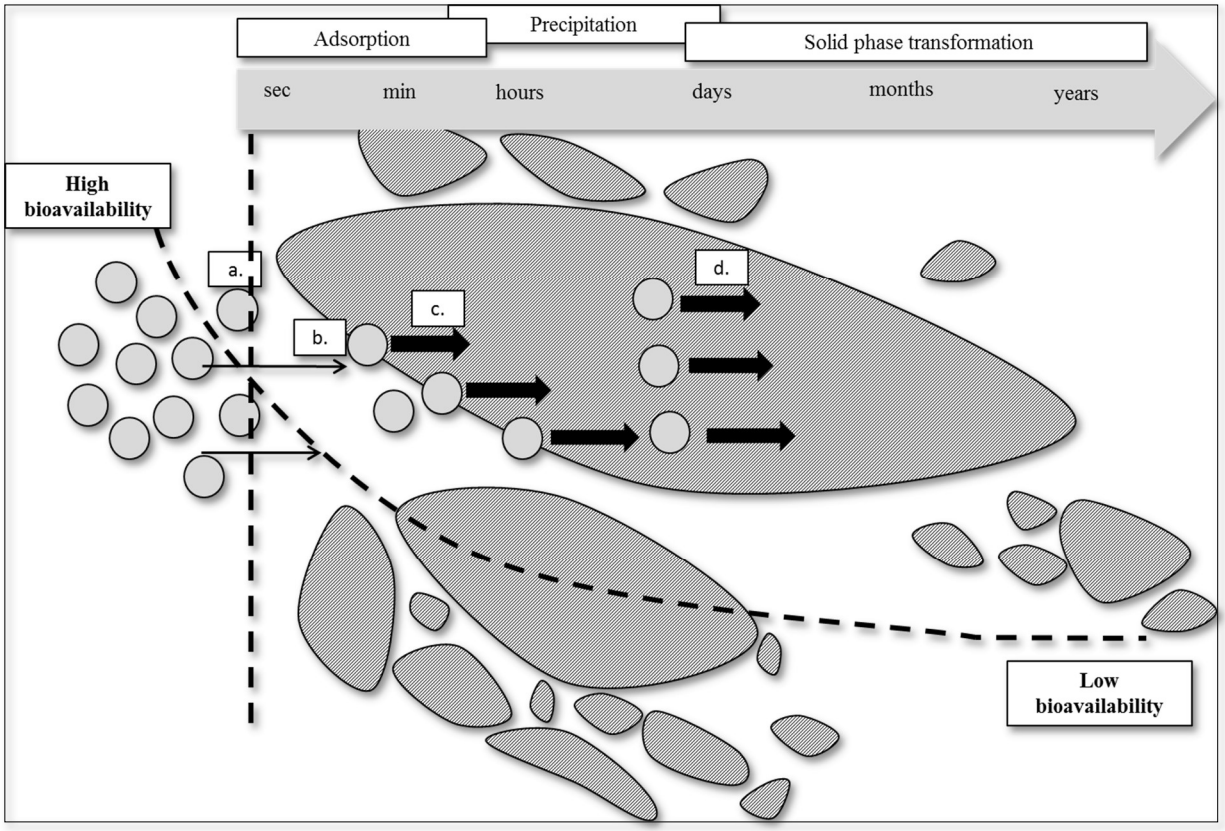


Figure 1.3. Conceptual diagram of the transportation and changes in sorption processes of metalloids with time in soil environments. Modified from Violante et al. (2008) and Naidu et al. (2008). The letters a) to d) represent the following processes: a) Metalloids crosses the solution-solid phase boundary and transported into a liquid filled soil macropore: b) During transport through the macropore, metalloids adsorb onto the surfaces of the soil particles via inner-sphere and outer-sphere complexation: c) With ageing, the initially adsorbed metalloids redistribute to the interior of the soil and d) The stabilization of surface precipitated metalloids through solid phase transformation recrystallization (Ostwald ripening).

Arsenic has been shown to have a strong binding affinity to Fe (hydr)oxides such as amorphous iron hydroxides, ferrihydrite and goethite with As(V) exhibiting a higher affinity than As(III) under most conditions (Pierce et al. 1982, Bowell 1994, Wilkie et al. 1996, Bang et al. 2004, Arai 2010, Violante 2010, Wilson et al. 2010). However, this adsorption behaviour is highly pH dependent for As(V) to Fe (hydr)oxides. In general, As(V) adsorption on to Fe/Al (hydr)oxides decreases with increasing soil pH (i.e. maximum adsorption under acidic conditions, with the adsorption affinity gradually decreasing with increasing pH between 6-10) (Arai et al. 2001, Arai 2010). In contrast, As(III) adsorption on to Fe/Al oxides gradually increases with increasing soil pH (from

pH 3.5 to pH 8-9) (Arai et al. 2001). During surface complexation, As(V) adsorption forms inner-sphere complexes with a bidentate or monodentate linkage the anion and surface of the Fe oxides and As(III) adsorption forms a combination of inner- and outer-sphere surface complexes with a bidentate binuclear linkage (Figure 1.4) by replacing -OH^- or -OH_2 groups from the surfaces of variable-charge minerals (Pedersen et al. 2006, Violante 2013, Qi et al. 2014).

Although the adsorption behaviour of Sb species has not been well investigated, some studies have showed a strong affinity of Sb species for Fe (hydr)oxides including amorphous Fe oxides, goethite, lepidocrocite and hematite (Leuz et al. 2006, Xi et al. 2013, Guo et al. 2014) and Mn (hydr)oxides (Wilson et al. 2010, Xi et al. 2010, Hockmann et al. 2014, Herath et al. 2017).

In general, Sb(V) adsorption to oxides and hydroxides increases with decreasing soil pH whereas Sb(III) adsorption is constant over a wide pH range (Leuz et al. 2006, Wilson et al. 2010, Guo et al. 2014, Hockmann et al. 2014). Similar to As, Sb forms inner-sphere surface complexes with bidentate mononuclear linkage during the adsorption on to oxides and hydroxides (Guo et al. 2014). Moreover, Sb(III) exhibits a higher affinity for oxides and hydroxides than Sb(V) (Wilson et al. 2010, Hockmann et al. 2014, Rouwane et al. 2016).

Phyllosilicates minerals

Phyllosilicate minerals are layers or sheets of linked units of tetrahedral silicate (SiO_4^{4-}). The adsorption of As and Sb on to phyllosilicates minerals such as illite, montmorillonite, kaolinite and clay minerals have been previously studied (Manning et al. 1997). Most of the clay minerals are found naturally on the earth surface and consist of silica, alumina, water and weakly bonded cations (i.e., Fe^{2+}, Mg^{2+}, Mn^{2+}) (Uddin 2017). Anionic adsorption to clay minerals frequently occurs through surface ligand exchange mechanisms. Clay minerals also play an important role in adsorbing As from natural environments and act similarly to metal oxides and hydroxides. For example, As(V) adsorption to clay minerals is high around pH 4-6 (with maximum adsorption at a pH of 5 and decrease with increasing pH above that range (Goldberg 2002)). However, As(III) adsorption exhibited parabolic behaviour with increasing pH between 3 to 9 (maximum adsorption around 8.5) (Goldberg 2002, Wang et al. 2006, Wilson et al. 2010).

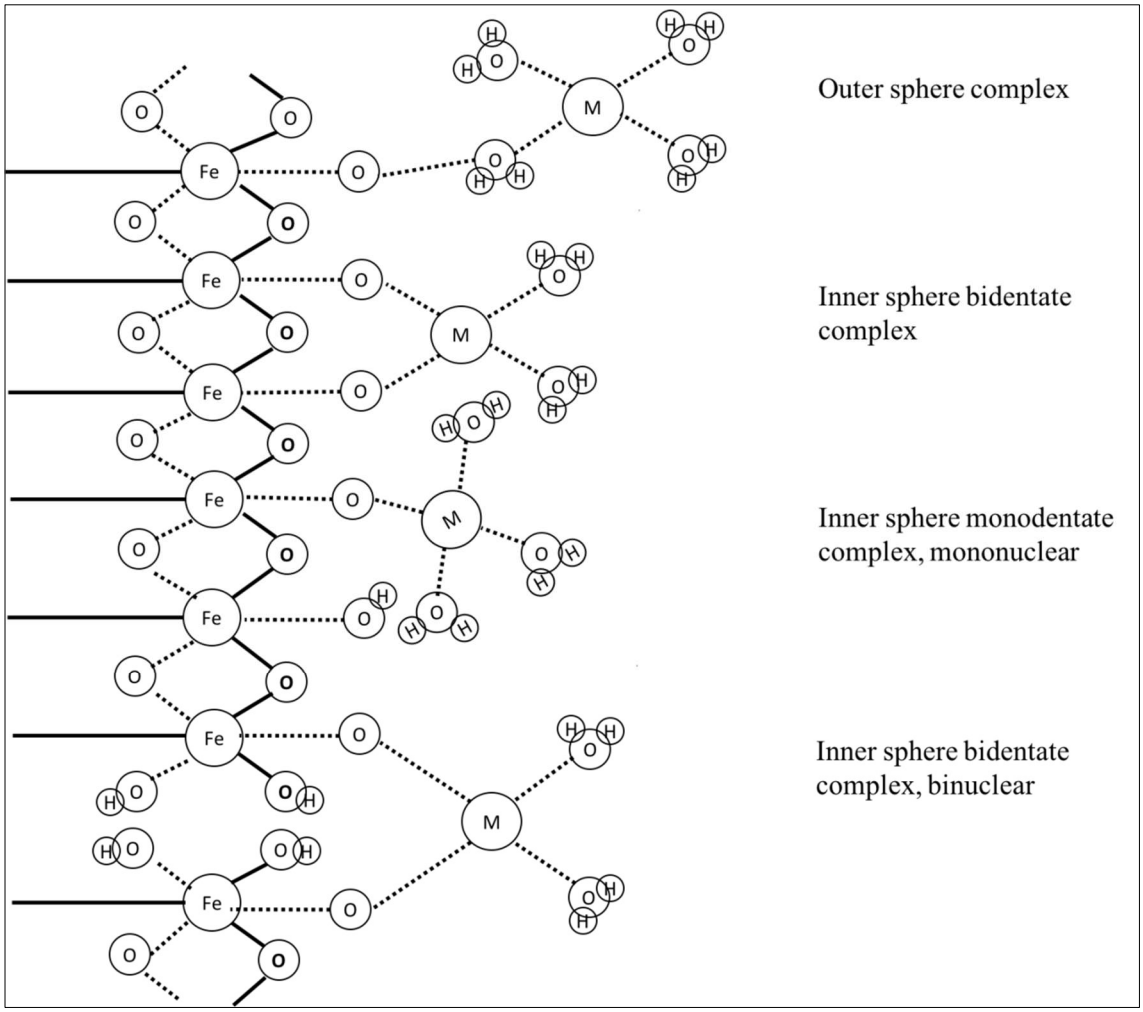


Figure 1.4. Formation of inner and outer sphere complexes of arsenic and antimony at the soil solution interface. Figure taken and modified from Goldberg et al. (2007).

Unlike As, both Sb(III) and Sb(V) bind weakly to clay minerals compared to Fe/Mn (hydr)oxides (Leuz et al. 2006). Further, Sb(III) shows a higher adsorption affinity to bentonite (a clay mineral) than Sb(V) (Herath et al. 2017). Bentonite is a major type of silicate clay mineral present in soils (Xi et al. 2011). Antimony(V) adsorption onto kaolinite (clay mineral) and Al silicate minerals is strongest at acidic pH (pH 3-4) and decreases slightly (~20%) with increases in pH up to 9.2 (Wilson et al. 2010, Xi et al. 2010, Herath et al. 2017).

Organic matter

Soil organic matter (SOM) refers to the organic compounds present in soil derived from decomposed plant/animal products, and can be a part of the dissolved or solid phase in

both aquatic and terrestrial environments (Sharma et al. 2011). The main fractions of SOM are fulvic acids (FA, soluble at all pHs), humic acids (HA, soluble at pH ≥ 2) and humin (insoluble components of SOM) (Hayes et al. 2017). Among these, FA and HA are important as they can bind to the soil and sediment surfaces, changing their physical, chemical and biological characteristics. The majority of dissolved soil organic matter (FA and HA) are negatively charged at neutral pH, protonated and uncharged at low pH and dissociated and negatively charged at higher pH (Wang et al. 2006). Both As and Sb show a lower affinity for adsorption to SOM compared to metal oxides and hydroxides (Tighe et al. 2005).

Both As(V) and As(III) can form stable aqueous complexes with FA and HA by binding to positively charged amino groups, with As(III) having a greater affinity for complexation than As(V) (Ko et al. 2004, Bauer et al. 2006, Wang et al. 2006). The maximum adsorption of As(V) and As(III) on humic acids is observed at pH 5.5 and 8.5, respectively (Wang et al. 2006). Antimony oxyanions have been shown to have a greater affinity to bind on to SOM compared to As oxyanions (Dousova et al. 2015). Recent studies have shown that the adsorption behaviour of Sb species to HA is pH dependent, with the adsorption affinity of both Sb(V) and Sb(III) decreasing with increasing soil pH. The maximum adsorption for Sb(V) was observed at pH < 7 (Tighe et al. 2005, Fan et al. 2019) and for Sb(III), a maximum adsorption was observed at pH 6 (Buschmann et al. 2004).

1.3.2 Competitive ligands

The adsorption of As and Sb on to sorption sites may be influenced by the presence of competitive ligands in soils. Organic ligands (such as FA and HA), inorganic anions (such as sulphate (SO_4^{2-}), phosphate (PO_4^{3-}), carbonate (CO_3^{2-}), nitrate (NO_3^-), hydroxyl (OH^-) and silicate (SiO_4^{4-})) and cations (such as Mg^{2+}, Ca^{2+} and Fe^{2+}) are known as the most important competitive ligands in soils (Jain et al. 2000, Caporale et al. 2016). These ligands can affect adsorption directly by competing for the number of available sites for adsorption and indirectly by changing the charge due to adsorption of anions and hence altering the electric potential of surface sorption sites (Jain et al. 2000, Caporale et al. 2016).

The effect of competitive ligands can significantly change with soil pH and an increase in anionic ligand concentration (John et al. 2018). Organic ligands can reduce metalloid

adsorption by forming soluble metalloid-organic complexes, or promoting adsorption by forming stable surface-metalloid-ligand complexes (Sharma et al. 2011). The dominant adsorption mechanisms of these ligands are known as ligand exchange and surface complexation between carboxyl/hydroxyl groups of organic matter and metal (hydr)oxides (e.g., Al and Fe (hydr)oxides).

In general, the presence of FA (between pH 3-8) and HA (between pH 6-9) decreases As(V) adsorption on Fe (hydr)oxides with FA being a better competitor than HA due to its smaller molecular size (Grafe et al. 2002, Weng et al. 2009, John et al. 2018). The presence of inorganic ligands also decrease As(V) and As(III) adsorption on to Fe/Al/Mn (hydr)oxides with more efficient desorption observed at higher pH (Caporale et al. 2016). The efficiency of As(V) desorption by anionic ligands in Fe/Al/Mn (hydr)oxides increased as follows; NO_3^- < SO_4^{2-} < CO_3^{2-} < SiO_4^{4-} < PO_4^{3-} (Meng et al. 2000, Genc Fuhrman et al. 2003, Guan et al. 2009, Zhang et al. 2009). In contrast, the presence of cationic ligands such as Mg^{2+} and Ca^{2+} increase the adsorption of As(V), which could be due to the reduction of the negative charge on the surface from absorption of cations onto the sorption sites (Zhang et al. 2009, John et al. 2018).

Limited information is available on Sb compared to As, but some studies have shown that the presence of organic ligands such as HA and other inorganic anions such as NO_3^-, CO_3^{2-}, SO_4^{2-} and PO_4^{3-} can partially decrease Sb(V) adsorption on minerals surfaces due to competitive adsorption effects (Wilson et al. 2010, Biver et al. 2011, Kolbe et al. 2011, Xi et al. 2011, Herath et al. 2017). The efficiency of Sb(V) desorption by anionic ligands in Fe/Al/Mn (hydr)oxides increased in the order NO_3^- < SO_4^{2-} < CO_3^{2-} < PO_4^{3-} (Biver et al. 2011) which is similar to As(V).

Farming practices often involve the addition of competitive ligands such as organic ligands (compost high in HA and FA) and/or inorganic anions in the form of fertilisers (PO_4^{3-}) to increase crop yield. Due to the influence of competitive ligands on adsorption/desorption of As and Sb to soil binding sites, this is likely to greatly affect the bioavailability of these metalloids in agricultural soils. However, limited information is available on the effect of the addition of competitive ligands such as fertilisers containing PO_4^{3-}, particularly on the bioavailability of As and Sb in co-contaminated soil. Therefore, further research is needed.

1.3.3 Precipitation and dissolution mechanisms

Precipitation

Arsenic and Sb can form insoluble compounds by precipitation or co-precipitation (Vu et al. 2003, Oorts et al. 2008). Iron and Al (hydr)oxides are considered important scavengers for As and Sb co-precipitation due their widespread distribution, large surface area and strong adsorption capacity. Common Fe-As precipitates in soil environments include scorodite ($FeAsO_4.2H_2O$), parasymplesite ($Fe_3(AsO_4)_2.8H_2O$) and phenmacosiderite ($Fe_4(AsO_4)_3(OH)_3.6H_2O$) (Aide et al. 2016). Further, at pHs below 4, As(V) in the soil solution can reduce to As(III) and co-precipitate with S^{2-} to form As_2S_3 (Sadiq 1997, Tiwari et al. 2013). Arsenic also co-precipitates with Mn to form $MnHAsO_4.8H_2O$ or highly insoluble minerals such as $Mn_3(AsO_4)_2.8H_2O$ (Kumpiene et al. 2008, Hartley et al. 2009). Antimony(V) can be precipitated as $Ca(Sb(OH)_6)_2$ at elevated Sb concentrations in calcareous soil, thereby controlling dissolved Sb in soil solutions (Oorts et al. 2008).

Reductive dissolution

Arsenate and Sb(V) strongly adsorb onto Fe/Al/Mn (hydr)oxides forming immobile complexes under aerobic conditions. However, flooded or waterlogged soils are usually anaerobic and can cause reductive dissolution of Fe(III)/Mn(IV) (hydr)oxides either biotically or abiotically (Mitsunobu et al. 2006, Rouwane et al. 2016), as shown in Figure 1.5. The biotic reduction of Fe(III) occurs by the release of enzymes from Fe(III) reducing microorganisms while abiotic reduction occurs via interactions with some organic compounds such as phenolics and chemicals with sulphydryl groups (Lovley 1997). The decrease in adsorption sites and surface site density as a result of reductive dissolution can promote As and Sb mobilisation in the soils leading to a greater risk of contamination to surface or ground waters. Further, the Fe(II) resulting from this process acts as an electron donor in the reduction of pentavalent forms to trivalent As and Sb species in the soil solution (Rouwane et al. 2016).

Both the release of adsorbed As(V) into the aqueous phase due to reductive dissolution of Fe(III)/Mn(IV) (hydr)oxides and reduction of As(V) to As(III) can lead to increased mobility of As in the soil (Sun et al. 2009, Wilson et al. 2010). However, the mobility of As(III) can be counteracted by As(III) co-precipitation with sulphur, forming insoluble sulphides (Signes-Pastor et al. 2007, Tufano et al. 2008, Yang et al. 2015). In a similar

manner, adsorbed Sb(V) can be released to the aqueous phase during the reductive dissolution of Fe(III)/Mn(IV) (hydr)oxides and reduced to Sb(III). However, unlike As, the mobility of Sb(III) can decrease due to strong adsorption onto Fe/Mn (hydr)oxides (Hockmann et al. 2014) at a pH ~7 (Rouwane et al. 2016). Limited information is available on the mobility and bioavailability of Sb under waterlogged conditions, especially when co-occurring with As. Competition for adsorption sites, ligands in precipitation processes, or release from the dissolution of metal oxides may affect As and Sb mobility and bioavailability under these conditions.

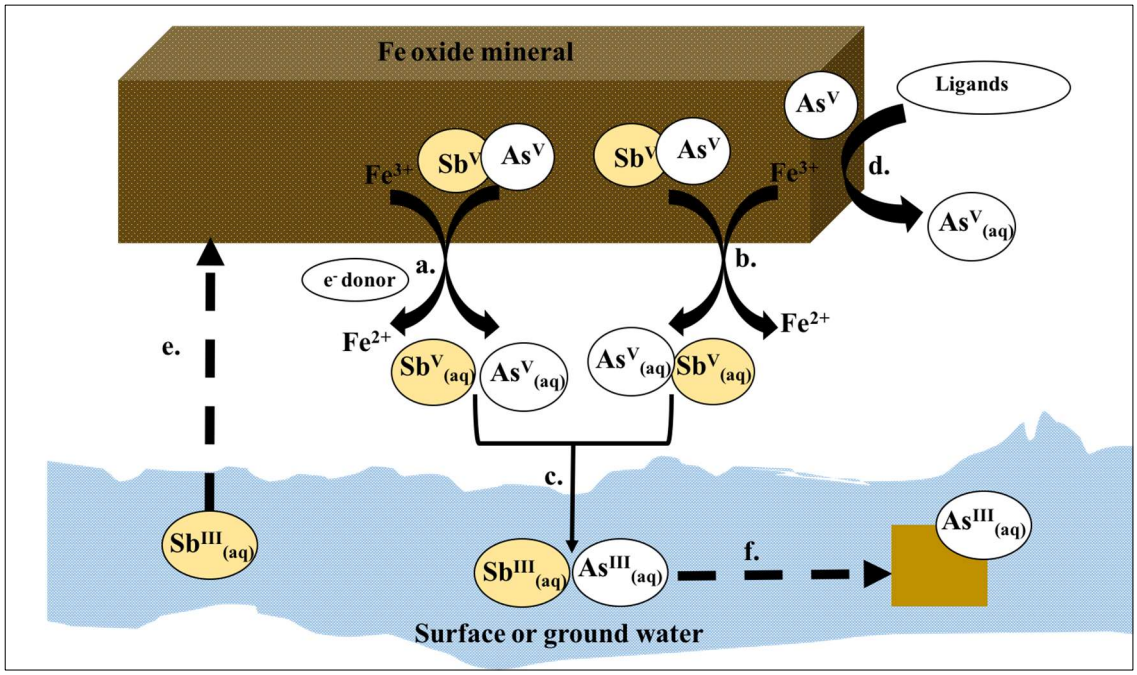


Figure 1.5. Conceptual diagram of arsenic and antimony mobilization from Fe(III) (hydr)oxides in minerals: a). the biotic reductive dissolution by microorganisms; b). abiotic reductive dissolution by soil organic matter releasing As(V) to the soil solution as a result of decreased soil sorption sites; c). reduction of As(V) and Sb(V) in the soil solution to As(III) and Sb(III); d). competition for binding sites by ligands; e). adsorption of Sb(III) on to Fe oxide mineral; f). co-precipitation of As(III) with sulphur.

1.3.4 Biotransformation in soils

The biotransformation of As and Sb by microorganisms is another factor which controls their fate in soils. Some microorganisms in soil can metabolize As and Sb oxyanions as electron acceptors in anaerobic respiration (Bowell 1994, Stolz et al. 2010, Finnegan et al. 2012). These metabolism processes include reactions such as methylation, demethylation, oxidation and reduction (Stolz et al. 2010). Methylation reactions can transform inorganic As species to methylated compounds such as methylarsonite (MMA(III)), dimethylarsinite (DMA(III)) and trimethylarsinite (TMA(III)), monomethyl arsonate (MMA(V)), dimethylarsinate (DMA(V)) and trimethylarsine oxide (TMAO(V)) and volatile compounds such as arsines (AsH_3), mono-, di- and tri-methylarsine (Stolz et al. 2010, Finnegan et al. 2012). Usually less than 5% of the total As in soils is present as organic species, with organic As(III) species in much lower concentrations compared to organic As(V) due to its high volatility (Huang et al. 2011, Finnegan et al. 2012). Similarly, inorganic Sb in soils can be methylated by microorganisms to form mono-, di-, and tri-methyl Sb compounds. However, this occurs less extensively than it does with As (Filella et al. 2009).

1.4 Plant uptake of As and Sb

1.4.1 Accumulation in agricultural plants

Arsenic and Sb are considered as nonessential to plants, but their bioavailable fraction can be readily accumulated by the plant roots posing a greater risk to human health through dietary exposure. The degree of bioavailability is directly influenced by soil conditions including soil pH, ageing, redox conditions and the presence of competitive ligands (such as PO_4^{3-}) and cations. Particularly, high As and Sb accumulation in plants occur in mining areas (Tschan et al. 2009, Zeng et al. 2015). For example, Baroni et al. (2004) found up to 540 and 216 mg/kg of As concentrations in roots and leaves of *Mentha aquatica,* respectively grown in mining dump (total soil As concentration; 899 mg/kg). Up to 1367 mg/kg of Sb concentrations have been found in the basal leaves of *Achillea ageratum* grown in Sb abandoned mining area (total soil Sb concentration; 9200 mg/kg) (Baroni et al. 2000). In comparison with these studies, low average concentrations of As (2.23 mg/kg) and Sb (24.53 mg/kg) concentrations were found in vegetables grown near XKS mine site, China where total As and Sb concentrations in soil were 172 and 2641 mg/kg, respectively (Zeng et al. 2015).

The ability of As and Sb to accumulate in plants also varies widely between plant species and family (Laizu 2007, Saldana-Robles et al. 2018). In support of this, Casado et al. (2007) showed among the plant species tested in their study, highest As accumulation found in *Tymus zygis,* while Sb accumulation found in *Thymus mastichina L*. Zeng et al. (2015) found differences in As and Sb accumulation within different vegetable types, with highest As accumulation by *Allium fistulosum* L. and Sb by *Coriandrum sativum,* while lowest concentrations of both metalloids by *Brassica pekinensis* L. However, the risk of As and Sb accumulation in edible plants depend on the vegetable types (underground and above ground vegetables) as they have different tendencies to transport into plant parts which is dependent on the accumulation ability and retention capacity of the plant in question. In general, As and Sb accumulation to plant parts decreases in the order of roots > shoots > leaves > fruits (Abedin et al. 2002, Dahal et al. 2008, Bhattacharya et al. 2010, Okkenhaug et al. 2012, Dutta et al. 2016, Ngo et al. 2016).

Different As and Sb species have different accumulation patterns in plants. The common As species present in soil are As(III), As(V), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (as discussed in Section 1.2.3). The species of As will influence the amount of As accumulated by various plant species. For example, Carbonell-Barrachina et al. (1999) showed that MMA has a higher accumulation potential in radish roots and shoots compared to other As compounds. In another study, Carbonell-Barrachina et al. (1999) showed that the total amount of As in turnip plants increased in the order of MMA < DMA < As(III) < As(V). In regard to Sb, the common species present in soil are SbIII), Sb(V), monomethylantimonate (MMSb), dimethylantimonate (DMSb) and trimethylantimonate (TMSb) (Mestrot et al. 2016). Shtangeeva et al. (2012) showed that different forms of Sb had various abilities to transport into plant parts. For example, wheat had a higher affinity for Sb(III) whereas rye had a higher affinity for Sb(V). Tschan et al. (2010) grew sunflower and maize in Sb(III) and Sb(V) spiked soil and showed that sunflowers have a higher affinity for Sb(V) whereas speciation of Sb had no influence on Sb uptake in maize.

Among methylated Sb species, TMSb was observed to be more mobile than methylated As(V) species in many soils found in mine tailings (Mestrot et al. 2016). Mestrot et al. (2016) investigated TMSb aboveground parts of ryegrass, however, it was not clear if methylation occurs in the plant or in the nutrient solution.The tolerable daily intake (TDI) of 129 µg/day has been provided for As (The Joint Food and Agricultural

Organization/World Health Organization (WHO) Expert Committee on Food Additives (JECFA)) while for Sb, this has been provided as 360 µg/day (WHO)(Wu et al. 2011). However, daily dietary intakes of these metalloids for the communities near mining area may exceeded these limits. For example, the daily dietary intake of As and Sb has been reported as 107 and 554 µg/day (for adults with body weight of 60kg) in the residents live near XKS mine, China (Wu et al. 2011). Among these, consumption of rice and vegetables (including tuber and leafy vegetables) contributed 63% and 18%, respectively of the total daily dietary intake of As while Sb contributed 33% and 26%, respectively (Wu et al. 2011).

Leafy vegetables are a staple in the diets of populations around the world. They are easy to grow, with some studies showing they are capable of growing in As and Sb contaminated soils with greater accumulation compared to other vegetables (Fu et al. 2016). This could lead to a potential risk of dietary exposure of As and Sb to humans. It has been reported that leafy vegetables have a higher accumulation factor compared to tuber, fruit and leguminous vegetables (Fu et al. 2016, Wu et al. 2019). The concentrations of As in leafy vegetables have been reported to be 0.55 mg/kg in edible parts of onion leaves, 0.1-1.53 mg/kg in water spinach, 0.49 mg/kg in celery and 0.45-1.12 mg/kg in spinach (Das et al. 2004, Agrawal et al. 2007, Dahal et al. 2008). There is limited data available on Sb accumulation in leafy vegetables.

Rice is a major food crop for half of the world's population, mainly in South-East Asia, including Bangladesh, West Bengal, China, Taiwan and Thailand where elevated As concentrations in soils and groundwater have also been reported (Meharg et al. 2002). In many countries, As concentrations in both white and brown rice range between < 0.01-2.05 mg/kg in Bangladesh, 0.31-0.70 mg/kg in China, 0.03-0.044 mg/kg in India, < 0.10-0.76 mg/kg in Taiwan, 0.11-0.66 mg/kg in the United States, 0.03-0.47 mg/kg for Vietnam, and 0.08-0.38 mg/kg for Italy and Spain (Meharg et al. 2008, Zavala et al. 2008, Bhattacharya et al. 2010). Antimony concentrations in brown rice near mining site ranged between 1.987-10.320 mg/kg in China (Fan et al. 2017). Considering the whole rice plant, the As and Sb accumulation in different parts decreased in the order of root > shoot > husk > grain (Wu et al. 2019). Moreover, in co-contaminated mining soil, despite the lower As concentration in soil, more As was accumulated in rice grains compared to Sb (Wu et al. 2019). Yet, more information is essential to understand the simultaneous accumulation of As and Sb in different parts of rice.

1.4.2 Uptake mechanisms

Arsenic and Sb in soils are taken up by plants via plant roots. The rate of their uptake and accumulation mainly depends on the surrounding soil characteristics which determine As and Sb speciation, and the plant species. Arsenic and Sb uptake mechanisms primarily depend on their chemical forms present in the soils, as plant roots have specific pathways and transporters to take up specific As and Sb species (Finnegan et al. 2012, Li et al. 2016). The main As species available for plant uptake are As(V), As(III), MMA, DMA (Li et al. 2016). In aerobic soils, As(V) transports across the root plasma membrane via inorganic phosphate transporters (PHT) due to their similar chemical structures (Finnegan et al. 2012, Li et al. 2016, Souri et al. 2017, Abbas et al. 2018) (Figure 1.7). There are over 100 phosphate transporters in the phosphate transporter 1 (PHT1) family and many of them present in plant roots have different selectivity's for arsenate and phosphate (Zhao et al. 2009). For example, in *Arabidopsis thaliana* PHT1;1 and PHT1;4 play a significant role in P(V) and As(V) accumulation from soils (Zhao et al. 2009, Li et al. 2016). The uptake of As(V) via phosphate transporters suggests that increases in phosphate concentrations in soil naturally or via the addition of phosphate based fertilisers will influence As uptake into plants through competition for the phosphate transporter. Thus, until now, some studies reported that As uptake in plants increased with increase of phosphate concentration in soil while other studies showed that increase of phosphate had no influence on As uptake (Quaghebeur et al. 2004, Pigna et al. 2009, Xenidis et al. 2010, Klaber et al. 2014, Zhang et al. 2017, Anawar et al. 2018).

Arsenic (III) is transported into the root cells via nodulin 26-like intrinsic protein (NIP) aquaglyceroporin channels (Mukhopadhyay et al. 2014, Souri et al. 2017, Abbas et al. 2018), along with water, glycerol and small neutral molecules and through the silicic acid transporters Lsi1 and Lsi2. In rice, the silicic acid transporters Lsi1 and Lsi2 are known as the major influx and efflux transporters of As(III) in both the exodermis (unicellular cell layer at the outer surface of the root) and endodermis (unicellular cell layer which separate the central cylinder of the root from the cortex), respectively (Schreiber et al. 2001, Ma et al. 2008, Li et al. 2016) (Figure 1.7). Abbas et al. (2018) reported that As(III) can move in both directions between root cells and soil solution depending on the As(III) concentration, as NIP and silicic acid transporters are bidirectional, whereas, As(V) can move only in one direction, as PHT transporters are unidirectional. Methylated As species like MMA and DMA have mainly been found in flooded soils and can also be taken up

by plants, but generally less efficiently than inorganic As(V) or As(III) (Zhao et al. 2009). Further, in rice roots, undissociated MMA and DMA have been shown to be taken up by Lsi1 transporters (Li et al. 2009).

The uptake mechanisms of As has been extensively studied, less information is available on Sb uptake mechanisms. Due to the different structures between the oxyanions of As(V) and Sb(V), it is assumed that they are not taken up through the same uptake pathway. Ji et al. (2018) and Tschan et al. (2008) suggested that Sb(V) uptake is primarily associated with the apoplastic pathway (flowing through cell wall to cell wall) which depends on the concentration gradient of ions between plant root and the soil (Figure 1.7). Similar to As(III), Sb(III) is assumed to be taken up passively through aquaglyceroporins across the root membrane, as $Sb(OH)_3$ (Figure 1.7) (Mukhopadhyay et al. 2014, Tisarum et al. 2015, Ji et al. 2018). Previous studies, however, have shown that aquaglyceroporin channels have different affinities for As(III) and Sb(III) uptake. For example, Meharg et al. (2003) showed that aquaglyceroporin channels in rice plants had a greater affinity for As(III) than Sb(III) as the uptake of Sb(III) was reduced by 50% in the presence of As(III) in rice growing for 20 min in water containing 0.1 mM As(III) (Meharg et al. 2003). However, whether there is competition between As and Sb for these uptake pathways in co-contaminated soils is still not clear.

1.5 Toxicity and detoxification mechanisms of As and Sb to plants

1.5.1 Toxicity to plants

Arsenic and Sb are non-essential to plant growth and their elevated concentrations can cause toxicity. Inorganic forms of As and Sb are generally considered to be more toxic than organic forms (Wilson et al. 2010, Joseph et al. 2015). However, this is not always the case and may be plant specific as DMA has been shown to be more toxic than As(V) to the germination rates and grain yields of wheat (Duncan et al. 2017). A species sensitivity distribution for 27 plant species exposed to As contaminated soils showed a reduction in plant growth by 10% and 50% at total soil concentrations of 7.83 and 25.27 mg/kg, respectively (Sun et al. 2012). In cucumber and kidney beans, the As concentrations which caused 50% reduction (EC_{50}) in growth indexes (growth indexes were measured using end points such as germination rate, root length, root biomass and plant height) were observed at 16.3 and 14.9 mg/kg, respectively. In soybean, tomato,

wild cabbage, green bean and rice EC_{50}'s ranged between 64-91 mg/kg with much greater EC_{50}'s were observed in ginger (225 mg/kg) and sweet potatoes (795 mg/kg) (Sun et al. 2012).

Toxic effects of As to plants include inhibited root extension and cell growth, reduced fertility, yield, fruit production and overall reduction in plant growth and biomass reduction (Finnegan et al. 2012). These toxic effects are mainly associated with the accumulation of AsO_4^{3-} in the cellular cytoplasm, which can disrupt phosphate-dependent metabolic processes by forming unstable adenosine di-phosphate (ADP) arsenate complexes which disrupts energy production in plant cells leading to cell death (Meharg et al. 2002, Garg et al. 2011, Finnegan et al. 2012). In the cytoplasm of plant root cells, As(V) can also be reduced to As(III) by arsenate reductase (AR) and bound by sulphydryl (-SH) groups of peptides in enzymes and tissue proteins, causing them to lose their functionality, also eventually leading to cell death (Meharg et al. 2002, Zhao et al. 2009, Garg et al. 2011, Abbas et al. 2018). Accumulation of As in plants leads to the generation of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2, which are strong oxidising agents (Abbas et al. 2018). These reactive oxygen species may damage biomolecules such as lipids, proteins and DNA (Garg et al. 2011), thus causing toxic effects to the exposed plant.

Only a few studies have investigated toxicity of Sb to plants; these include ferns, sunflowers and maize (Feng et al. 2008, Ortega et al. 2017). Antimony toxicity mainly depends on its concentrations and speciation, and in general Sb(III) is known to be more toxic than Sb(V) (Zhou et al. 2018). Antimony is easily absorbed by plants and can induce toxic effects such as reduced growth and reduced synthesis of some metabolites (Zhou et al. 2018). For some plants exposure concentrations as low as 5-10 mg/kg have been shown to be toxic (Feng et al. 2011). Feng et al. (2013) and He (2007) reported that the NOEC for Sb was 65.5 and 54 mg/kg to rice and radish leaves, respectively. In another study, Tschan et al. (2009) showed that the shoot biomass of sunflower, maize and clover seedlings decreased to ~50% relative to controls at total soil Sb concentrations > 190 mg/kg. A 50% reduction in barley root elongation and lettuce shoot yield were also observed at total soil Sb concentrations 0.056 mol/kg (6810 mg/kg) and 0.062 mol/kg (7546 mg/kg) (Oorts et al. 2008).

Antimony in the form of Sb(III) has been shown to inhibit the photosynthetic electron transfer chain, the carbonic anhydrase activity of photosystem II and the glutathione

reductase activity of chloroplasts (Karacan et al. 2016, Ortega et al. 2017). A decrease in the biosynthesis of chlorophyll and carotenoids was also observed with increasing Sb concentrations (Ortega et al. 2017, Zhou et al. 2018). Such effects are likely to have toxic effects on plant species. The toxicity of Sb has been associated with increased ROS production as indicated by high malondialdehyde content (an indicator of the extent of lipid peroxidation) in ferns *Microlepia hancei* and *Cyclosorus dentatus* (Feng et al. 2013). Exposure to Sb can also simply disturb the uptake of essential nutrients from the soil and thereby affect their distribution in the plant (Feng et al. 2013). For example, Shtangeeva et al. (2011) demonstrated a significant decrease in the uptake of Ca^{2+} and Na^{+} in wheat (*Triticum aestivum*) seedlings after exposure to Sb.

1.5.2 The risks of As and Sb mixtures in the environment

Most toxicity assessments involve single contaminant exposures, yet most chemicals in the environment are present as mixtures. A growing body of evidence shows that chemical mixtures can pose a greater or lower risk than expected from individual exposures (Simmons 1995). The toxicity of chemical mixtures can be equal to the sum of the fractional toxic effects of individual components (additive) or may induce either higher (synergistic) or lower (antagonistic) toxicities (Wang et al. 2006, Li et al. 2014). Some contaminants may also be present at concentrations lower than what is expected to cause significant toxicity, such as the no observed effect concentration (NOEC), but may still alter the overall toxic response of other contaminants during exposure (Wang et al. 2006, Okkenhaug et al. 2012, Barać et al. 2015). Thus, combined toxicity should be considered when assessing contaminants. Recently, As and Sb have been identified as global pollutants which are also non-essential for plant growth (Vaculík et al. 2015, Herath et al. 2016). Although the toxic effects of As and Sb are well documented during their individual exposures (Sun et al. 2012) little is known about their combined toxicity when present in the co-contaminated soil.

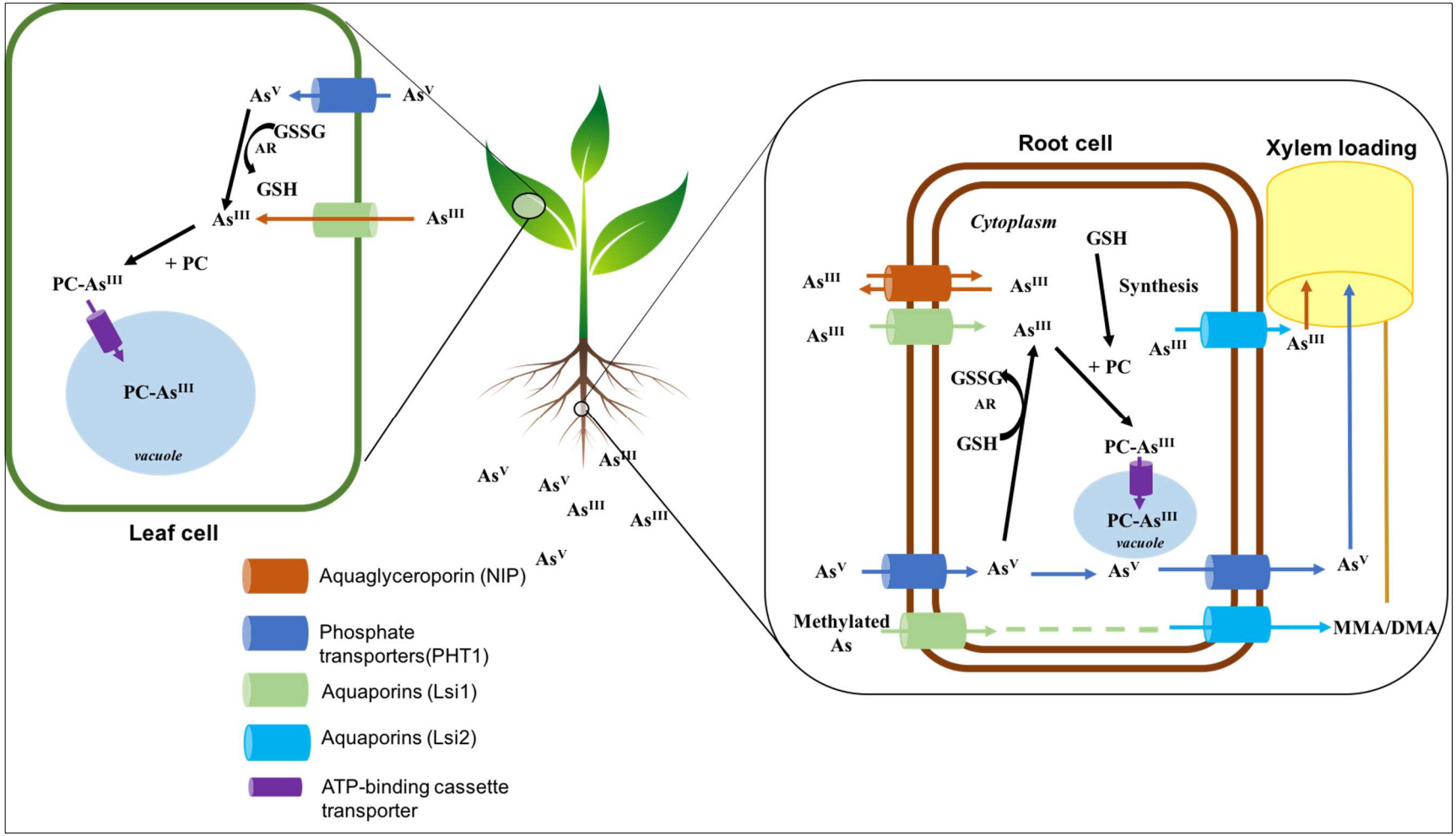


Figure 1.6. Uptake, transport and detoxification mechanisms of arsenic in plants under aerobic and anaerobic conditions (detailed uptake mechanisms are discussed in Section 1.4.2 and 1.5.1).

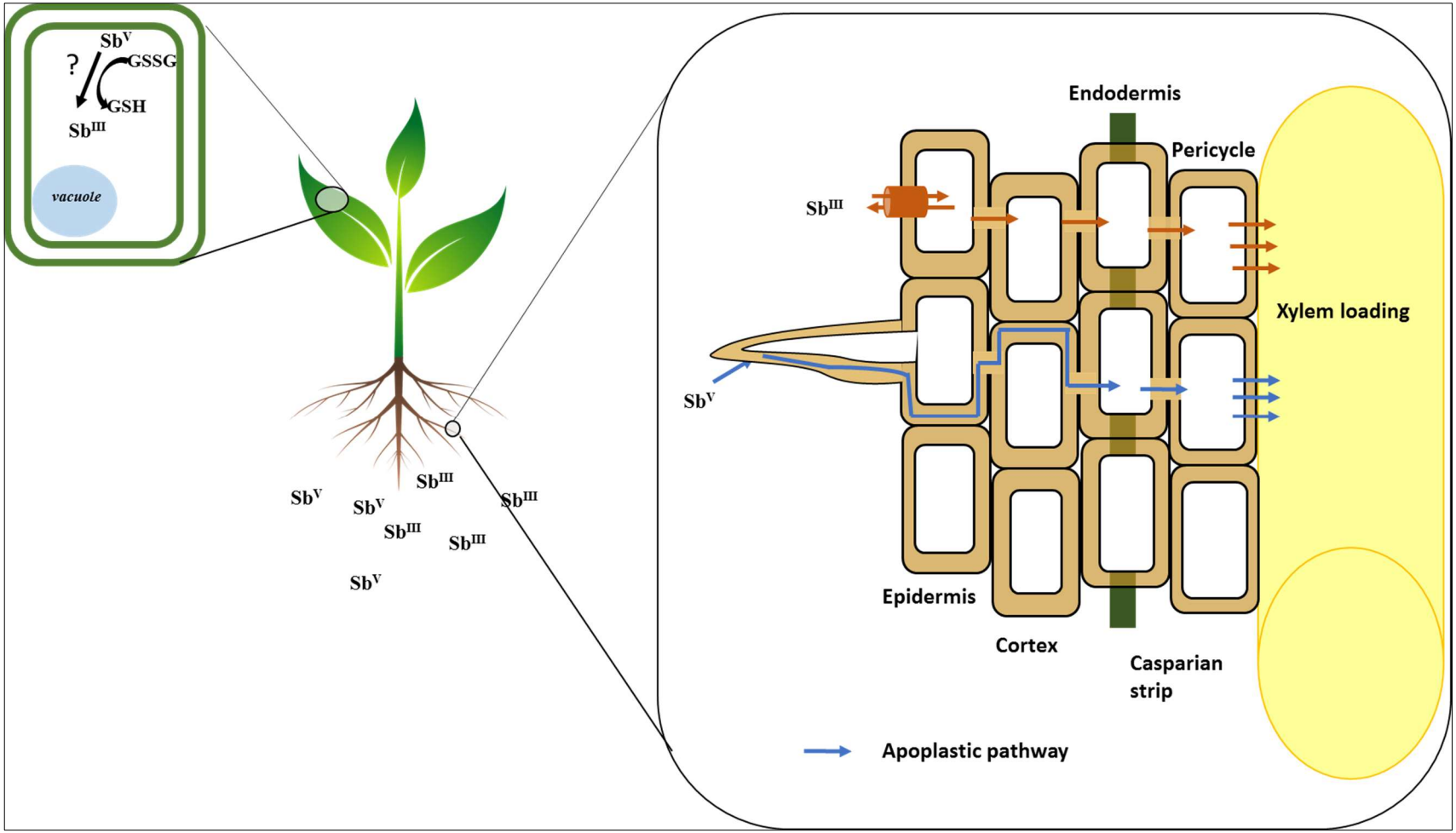


Figure 1.7. Uptake and transport of antimony in plants under aerobic and anaerobic conditions (detailed uptake mechanisms are discussed in Section 1.4.2).

1.5.3 Plant detoxification mechanisms

Arsenic tolerance and detoxification in plants is associated with many mechanisms, such as chelation, compartmentalization, biotransformation and cellular repair (Pigna et al. 2015). The main As detoxification mechanism in plants is the reduction of As(V) to As(III) (by AR) followed by complexation of As(III) with phytochelatin (PC) and sequestration in vacuoles or in the xylem via silicic acid transporters (Figure 1.6) (Garg et al. 2011, Zheng et al. 2012, Pigna et al. 2015). The efflux of As(III) from the root cells have less tendency to bind with thiol groups and is known as another detoxification mechanism (Abbas et al. 2018). Under anaerobic (waterlogged) conditions, the Fe(II) in the rhizosphere is oxidized to form iron plaques (by O_2 released from the roots) has been shown to have high affinity for As(V) which prevents its uptake, reducing its toxicity (Ren et al. 2014).

The main Sb detoxification mechanisms in Sb-tolerant plants include the production of antioxidative enzymes such as peroxidase, catalase and ascorbate peroxidase which are known as the H_2O_2 scavengers in plants (Feng et al. 2008, Chai et al. 2016). These are mainly produced in respond to excessive ROS species in the plant tissues. An increase production of phenolic compounds such as flavonoids has also been observed in response to oxidative stress of Sb (Ortega et al. 2017). Similar to As, phytochelatins are assumed to detoxify Sb in plants (Feng et al. 2013). However, less information is available on the role of PC on Sb detoxification.

The toxicity of As and Sb in co-exposures is not well understood because most studies have investigated their toxicity and detoxification mechanisms under single-exposure conditions. However, they often co-occur (discussed in Section 1.2) so more research is needed to understand whether As and Sb lead to interactive toxic effects.

1.6 Factors affecting As and Sb in agricultural environments and key gaps in our knowledge

A range of environmental and anthropogenic factors affect As and Sb bioavailability in soil (Section 1.3). However, not all of these are relevant to agricultural environments where soils are carefully controlled to grow agricultural crops. The ageing time of As and Sb contaminants in soils, the co-contamination of As and Sb, the application of phosphate-based fertilisers and waterlogging of soils are some agriculturally relevant

factors that will affect As and Sb bioavailability and hence, accumulation and toxicity.

The effect of soil ageing on As and Sb bioavailability is essential to estimate the magnitude of potential environmental and human health problems caused by these metalloids over the time. Many of the processes discussed in Section 1.3 are interlinked and lead to a decrease in As and Sb bioavailability with increase of soil ageing (metalloid-soil residence time). This can decrease their accumulation and toxicity to plants grown in contaminated soils. Juhasz et al. (2008) showed that soil ageing decreased As bioavailability by ~25% in spiked soil (Red Ferrosol) aged for 12 months while Hammel et al. (2000) showed a decrease of Sb bioavailability by 7.8-8.7% in spiked soil aged for 6 months. These studies are mainly conducted on As and Sb individually contaminated (spiked) soils; however, previous studies have shown that metalloids in contaminated soil often occur as mixtures (Section 1.2) (He 2007, Li et al. 2014, Ngo et al. 2016, Doherty et al. 2017). Thus, it is essential to understand how soil ageing affects the bioavailability, accumulation and toxicity of As and Sb to agriculturally important crop species in co-contaminated soil over the time.

Arsenic and antimony assumed to have similar geochemical behaviour because of their similar oxidation states of III to V in soil and aqueous compartments (Section 1.2.3 and 1.2.4). Therefore, the competitive interactions between them may affect their bioavailability, accumulation and toxicity. Recent studies, however, suggest that As and Sb cannot be assumed to have similar geochemical behaviour due to their different coordination structures (Tschan et al. 2009, Wilson et al. 2010, Fu et al. 2016). Nevertheless, none of these studies have considered the effect of As and Sb on their bioavailability in co-contaminated soils. Different geochemical behaviours may cause changes to As and Sb accumulation and toxicity when present as a mixture. For example, by direct competition where the metalloids might compete for the same binding site or uptake pathway (Andrewes et al. 2000). Muller et al. (2013) and Feng et al. (2011) showed an increase in Sb uptake in the presence of As in the non-edible As hyperaccumulating brake ferns *Pteris vittata* and *Pteris cretica*. Further, Feng et al. (2015) observed different accumulation and subcellular distribution of As and Sb in four fern plants, *Pteris cretica*, *Pteris fauriei*, *Humata tyermanii* and *Pteris ensiformis*. However, the impacts and interactions of co-existing As and Sb on their accumulation and toxicity to agricultural plants in soil is not yet understood but is of concern because of the possible implications to human health through dietary exposure.

The rapid expansion of the global population has driven an increase in food production which has been met with a significant increase in the use of phosphate-based fertilisers. Phosphate is an essential macronutrient for plant growth and is present within essential biomolecules including nucleic acids, phospholipids and adenosine triphosphate (Schachtman et al. 1998). Concurrently, there is still use of agricultural lands contaminated by As and Sb mainly due to improper waste disposal, contaminated water irrigation and offsite migration from mining sites (Section 1.2). The similar chemical properties between PO_4^{3-} to AsO_4^{3-} means that they can substitute each other on soil binding sites (Section 1.3.2) and transporters for plant uptake (as discussed in Section 1.4.2). Some studies have shown that the AsO_4^{3-} and PO_4^{3-} compete for the same soil sorption sites resulting an increased As bioavailability (Anawar et al. 2018) and eventually increasing As accumulation in agricultural plants; i.e., wheat root and shoot, chickpea shoot, carrot and lettuce (Cao et al. 2004, Tao et al. 2006, Gunes et al. 2009, Pigna et al. 2009). However, this is not the case for Sb. Antimonate uptake did not appear to compete for the same soil sorption sites or occur via phosphate pathways. Feng et al. (2013) and Tschan et al. (2008) showed that the addition of PO_4^{3-} did not affect the uptake of Sb in maize and sunflower plants. Given that As and Sb co-occur in contaminated lands as discussed above, it is important to understand how their interactive effects on bioavailability, accumulation and toxicity change with the addition of PO_4^{3-}, particularly under environmentally realistic scenarios of As and Sb co-contamination. Further, there is little information about the impacts of soil PO_4^{3-} impact on Sb bioavailability and toxicity to agricultural plants.

Waterlogging is a common soil condition required to grown rice in paddy soils. This leads to a change in the soil's redox conditions. The change of soil redox conditions can greatly influence the chemical speciation of As and Sb. Different As and Sb species have different affinities to bind on to soil sorption sites, influencing their mobility and bioavailability in soils (Sections 1.3). For example; the lower binding affinity of As(III) and Sb(III) under waterlogged (anaerobic) conditions can cause them to become more mobile and bioavailable in the agricultural soil (Hockmann et al. 2014). This can cause more As and Sb to accumulate in agricultural plants grown in waterlogged conditions than under aerobic conditions, posing a greater risk. Our understanding of how such conditions alter As and Sb speciation and bioavailability is mostly contributed from studies under As and Sb individual exposures and the impacts of As and Sb co-

contamination within waterlogged soils on metalloid mobility, bioavailability, uptake and toxicity to agricultural plants is still unknown.

Therefore, further research is needed to understand the influence of soil ageing, co-contamination (As and Sb as a mixture), PO_4^{3-} concentration and waterlogging on the mobility and bioavailability of As and Sb in soils, and effects on the accumulation and toxicity of these metalloids to agricultural plants. This is especially important given the potential risks these metalloids pose to human health.

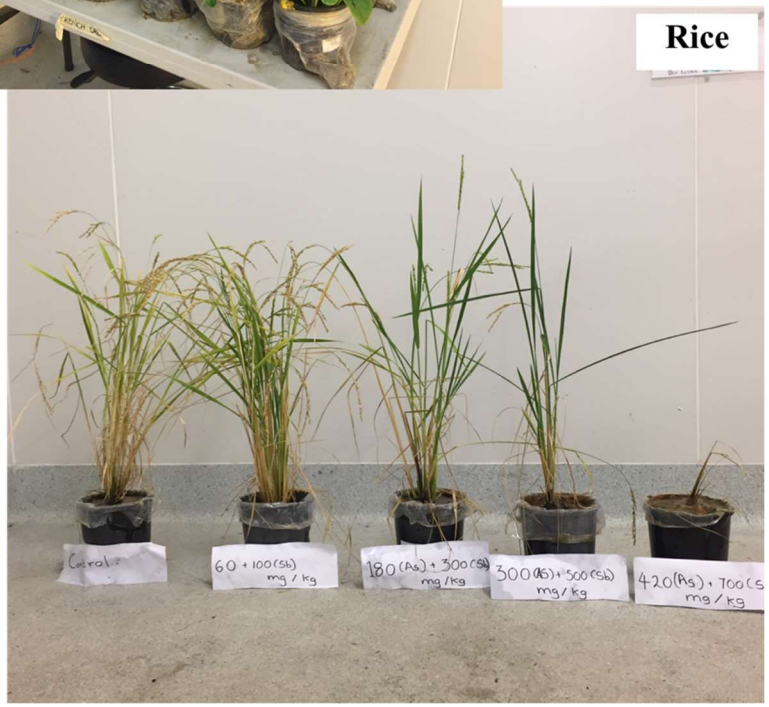
1.7 Aims and objectives

The aim of this research is to investigate the factors that influence the bioavailability and toxicity of As and Sb to agricultural plants grown in contaminated soils. This will be achieved by:

1. Investigating the impacts of soil contaminant ageing on the bioavailability, accumulation and toxicity of As and Sb on the agriculturally important plant water spinach (*Ipomoea aquatica*) grown in historically and recently contaminated soils (Chapter 3). The bioavailable As and Sb fractions are compared to total soil and accumulated metalloid concentrations to determine the influence of ageing on bioavailability and accumulation. The effects of ageing on the toxicity of As and Sb to *I. aquatica* is also assessed by measuring root and shoot growth (dry mass and length) and photosynthetic efficiency.
2. Determining the interactive effects of As and Sb on metalloid bioavailability, toxicity and accumulation by exposing *I. aquatica* to As and Sb individually and as a mixture at concentrations representative of contaminated soils. Plant toxicity endpoints such as shoot/root dry mass, length and chlorophyll *a* content of leaves along with accumulation data were compared with the total and bioavailable As and Sb concentrations in the contaminated soils (Chapter 4).
3. Investigating the influence of increased soil PO_4^{3-} concentrations on As and Sb bioavailability, accumulation and toxicity to the commonly consumed leafy vegetable, choy sum (*Brassica chinensis var. parachinensis*) grown in As and Sb individually and co-contaminated soils. The toxicity was assessed by measuring shoot and root dry mass and shoot and root length (Chapter 5).
4. Investigating the behaviour of As and Sb in soil-rice plant systems across its

whole growth period using a rice (*Oryza sativa*) bioassay grown in individually and co-contaminated As and Sb soils. The toxic effects of As and Sb on the growth of rice will be assessed by comparing the bioavailable concentrations of Sb and As with endpoints such as dry mass of root, shoot and grains and lengths of roots and shoots. This Chapter will also assess the competitive effect of co-contamination on As and Sb accumulation and translocation from soil to rice plants by analysing the concentrations of Sb and As in rice tissues (Chapter 6).

Chapter 2. General Methods



2.1 General washing methods and analytical reagents

All solutions were prepared with analytical grade chemicals and high purity water (distilled deionized water, 18.2 MΩ.cm, Merck Millipore, **Table A1.1**). All plastic and glassware were either new or acid washed in 10% (v/v) HNO_3 using a Smeg GW3060 glassware washer. High purity water was used for the preparation of all solutions.

2.2 Test soils

Uncontaminated soil was collected from the surface horizon (5-30 cm) from Wollongong area (NSW, Australia). The soil samples were air dried, crushed and sieved to <2 mm.

Historically contaminated soil used in Chapter 3 was collected from a decommissioned antimony processing facility in Urunga, NSW (Australia) and bioassay concentrations were prepared by mixing fields collected contaminated and control soils at various ratios. Recently contaminated soil used in Chapter 4, 5 and 6 were prepared by spiking uncontaminated soil (<2 mm) with As(V) (sodium arsenate) and Sb (V) (potassium hexahydroxoantimonate) solutions freshly prepared in high purity water to establish three concentration series; $As_{(Individual)}$, $Sb_{(Individual)}$ and $As + Sb_{(Combined)}$. The nominal concentrations of the test soils used are provided in each chapter. The ratio and concentration range of As and Sb used in thesis are based on the studies of Ngo et al. (2016) and Okkenhaug et al. (2011). The spiked soils were vigorously mixed a few times to achieve a homogeneous soil mixture. The spiked soil was left to equilibrate in a greenhouse at ambient temperature for 14 days, then air dried, crushed and sieved to <2 mm. Subsamples for total metalloid analysis and soil characteristics were collected from each concentration (n=3) on day 14 prior to commencement of bioassays. In this thesis, $As_{(Individual)}$, $Sb_{(Individual)}$ and $As + Sb_{(Combined)}$ contaminated bioassays are referred to as the treatments from this point.

2.3 Test plant species

A summary of the methods is presented in Figure 2.1. This thesis used three different plant species: water spinach (*Ipomoea aquatica*), choy sum (*Brassica chinensis* var. *parachinensis)* and rice (*Oryza sativa*) to evaluate various patterns of As and Sb accumulation and toxicity in plants. In Chapter 3 and 4, the bioassays were established using *I. aquatica*, commercially purchased from Green Harvest Organic Gardening

Supplies Pty Ltd. In Chapter 5, choy sum bioassays were established from commercially purchased Johnsons Choy Sum Seeds. In Chapter 6, *O. sativa* bioassays were established from seeds purchased from Herbalistics.

2.3.1 Establishment of the bioassays

Prior to each bioassay, dead seeds were isolated by soaking seeds in high purity water for ≥ 1 h, those that floated to the surface were removed.

Tests were prepared in triplicate by adding ~1.4 kg of equilibrated soil to circular, commercial plastic pots with dimensions of 10 (diameter) x 15 (height) cm. The base of each pot was lined with 2 mm weaving nylon mesh and a high-density polyethelene bag was placed around the pot to contain drainage. A nutrient solution was added to provide 0.15 g N/kg, 0.1 g P/kg and 0.04 g K/kg, as per typical agricultural practices to avoid nutrient deficiencies (26). Twelve seeds were sown in each pot (Day 0) and allowed to germinate. On day 14, the six healthiest seedlings were selected for the bioassay, and others were removed. The bioassay continued within a controlled environment (Thermoline Scientific Incubator with a day:night cycle of 14:10 h at 30˚C and 25˚C, respectively, and light intensity at 500 µmol m^{-2} s^{-1}). Incubators automatically watered twice (10 min each) daily to maintain a soil water holding capacity of ~60%. A summary of bioassay establishment is shown in Figure 2.1.

2.3.2 Toxicity endpoints

After bioassays were terminated, plants were harvested, rinsed with high purity water to remove absorbed soil and wet mass was determined. Plants were sectioned into shoots and roots using a stainless-steel knife, and the lengths were measured. Both shoots and roots were oven dried at 60 ˚C for 2 days and dry mass was determined.

2.4 Soil physical and chemical characteristics

2.4.1 Soil particle size

The particle size distribution of the soil concentration series was analysed using a Mastersizer 2000 (Malven Instrument Ltd, Worcestershire, United Kingdom). The Mastersizer 2000 uses a combination of the theories of Mie scattering and Fraunhofer diffraction. A 4 mW (output power), 632.8 nm (beam wavelength) red laser and 0.3 mW

(output power), 470 nm (beam wavelength) blue laser in a convergent beam setting was passed through a sample of soil suspended in water. The patterns of diffraction and scattering were analysed to determine the particle size distribution. Particle size was reported as the mean size and silt/clay fraction.

2.4.2 Soil pH

Soil (5g) was mixed vigorously (shake for 2 h) with high purity water in a 1:5 soil to high purity water mass ratio and allowed to stand for few minutes to settle the suspension in the bottom ((Buurman et al. 1996). The pH of the supernatant was measured utilising a pH meter (Thermo Scientific Orion 5-star bench top meter with ATC probe and ROSS Ultra pH electrode). Prior to use, the meter was calibrated with buffer solutions at pH 4 (Orion, 910104) and 7 (Orion, 910107). Distilled deionized water was used to rinse the electrode between measurements and stored in 3 M KCl storage solution.

2.4.3 Soil moisture content

The weight of empty crucibles was measured. A known mass of wet soil (~1 g) was weighed into the crucible and oven dried at 105°C for 24 h. Then, the crucibles were transferred into a desiccator and cooled to room temperature. Finally, the crucibles were reweighed. Soil moisture was reported as a percentage using Equation 2.1 (Buurman et al. 1996):

$$\text{Moisture (\%)} = \frac{wet\ weight - dry\ weight}{wet\ weight} \times 100 \qquad \text{Equation 2.1}$$

2.4.4 Total organic matter (TOM) and organic carbon (TOC)

Loss on ignition (LOI) was used to determine total organic matter content in soils by calculating the weight loss between 105°C and 550 °C. A known mass of wet soil (~1 g) was weighed into the crucible and oven dried at 105°C for 24 h. The crucibles were removed from the oven and allowed to cool to room temperature and weighed. Then, the crucibles were further heated to 550°C for 2 h in a muffle furnace. Finally, the crucibles were dried and weighted. The TOM was calculated using Equation 2.2 (Ngo 2018).

$$\text{TOM (\%)} = \frac{weight\ at\ 110\ °C - weight\ at\ 550\ °C}{weight\ at\ 110\ °C} \times 100 \qquad \text{Equation 2.2}$$

The TOM content was converted to TOC content by using conversion factor of 1.724

(Schumacher 2002). The total organic carbon was reported as a percentage in relation to the dry soil mass using the Equation 2.3:

$$TOC(\%) = \frac{Total\ organic\ matter}{1.724} \quad \text{Equation 2.3}$$

2.4.5 Total kjeldhal nitrogen

Approximately 1 g of dried soil was weighed into an Erlenmeyer flask and diluted to 300 ml. Then, 25 ml of borate buffer and 6M NaOH was added until pH reached 9.5. The mixture was boiled off until white fumes appeared in the flask. After digestion, the solution was cooled and diluted to 300 ml with high purity water. Then, 50 ml of sodium hydroxide-thiosulphate was added, and steam distilled, where the generated ammonia was trapped in a boric acid solution. The formed ammonium was then titrated with sulphuric acid standardised with sodium carbonate solution (Rayment et al. 2011).

2.4.6 Extractable phosphorus

The Olsen's sodium bicarbonate extraction method was used analysed the extractable phosphorus (P) in test soil. This was determined by the extraction of ~2.5 g dried soil with 50 mL of 0.5 M sodium bicarbonate solution (extractant) for 30 min using reciprocating shaker at a minimum of 180 oscillations per minute. The solution was filtered through a Whatman filter paper (pore size-2.5µm) and filtrate was collected. The extracted phosphate was then analysed colourimetrically with an acidified solution of ammonium molybdate containing ascorbic acid and a small amount of Sb (Jones Jr 1999).

2.4.7 Maximum water holding capacity (MWHC)

Approximately 20g of dried soil was placed in a funnel with Whatman filter paper and the funnel was held in a container of water. Then water was added until the soil is saturated and allowed to stand for 2 h. The funnel was removed and allowed to drain overnight. The wet weight of the filter paper was measured by performing a blank measurement simultaneously. The MWHC of soil was calculated using Equation 2.4 (Ngo 2018):

$$MWHC\ (\%) = \left(W_{wet\ soil+wet\ f.\ p.} - W_{wet\ f.\ p.} - W_{dry\ soil}\right) x\ 100\%/W_{dry\ soil} \quad \text{Equation 2.4}$$

Where $W_{wet\ soil}$ is the mass of the wet soil and filter paper after drainage, $W_{wet\ f.\ p.}$ is the mass of the wet filter paper, and $W_{dry\ soil}$ is the mass of dry soil.

2.5 Soil and plant As and Sb concentrations

2.5.1 Sequential extraction procedure (SEP)

The sequential extraction procedure (SEP) utilised in this investigation was derived from Wenzel et al. (2001) and is summarised in Table 2.1. Analytical-grade chemicals including ammonium sulphate $(NH_4)_2SO_4$, ammonium orthophosphate $(NH_4)H_2PO_4$, ammonium oxalate and ascorbic acid were used for the sequential extraction procedure. Soil (~1 g) was combined with 25 ml of the relevant extractant, mixed by rotation for the times specified and then centrifuged (15 min, 3000 g). The final extraction (targeting the crystalline Fe and Al oxide phases) was conducted in a water bath (96°C for 30 min). Two of the extraction steps included a wash step, which involved mixing the soils with 12.5 ml of the appropriate extractant (10 min, dark), centrifugation and then combining it with the original supernatant for analyses. The bioavailable As and Sb soil fraction is operationally defined in this thesis as the sum of the non-specifically bound (SO_4^{2-} extractable) and specifically bound (PO_4^{3-} extractable) metalloid fractions, and will be referred to herein as the SEP-bioavailable soil fraction (Wenzel et al. 2001). It is noted that the SEP-bioavailable soil fraction is an approximation of the bioavailable fraction which does not necessarily take into account factors which may affect bioavailability to specific plants including time frames of extractions and specific plant-soil interactions.

2.5.2 Total concentration in soil

Soils were digested as per EPA method 3051 (EPA, 2007). Dried soil (0.2 g) was weighed and digested in Conc. HNO_3 (65% HNO_3 Suprapur, Marck Milipore, Australia) and HCl (37% HCl Suprapur, Marck Milipore, Australia) at a ratio of 1:3 (v:v) overnight and then heated in a MARS 6 microwave digestion system for at 175ºC (15 min, ramp time and 5 min, holding time). Digests were diluted with high purity water to 2% acid content prior to analysis.

Table 2.1. Sequential extraction procedure derived from Wenzel et al. (2001).

Soil binding target phase	Extraction and soil to solution ratio	Conditions	Additional wash
Ion Exchangeable	0.05 mol/L $(NH_4)_2SO_4$ (1:25)	4 h shaking, 20°C	
Weakly Labile	0.05 mol/L $(NH_4)H_2PO_4$ (1:25)	16 h shaking, 20°C	
Bound to amorphous Fe and Al oxides	0.2 mol/L ammonia oxalate buffer; pH 3.25 (1:25)	4 h shaking, in the dark, 20 °C	0.2 mol/L ammonia oxalate buffer; pH 3.25 (1: 12.5*), 10 min shaking in the dark
Bound to crystalline Fe and Al oxides	0.2 mol/L ammonia oxalate buffer + 0.1 mol/L ascorbic acid; pH 3.25 (1:25)	30 min in a water basin at 96 $\pm$ 3 °C in the light	0.2 mol/L ammonia oxalate buffer; pH 3.25 (1: 12.5*), 10 min shaking in the dark
Residual	Difference in sum of fractions and total metals analysis		

* Soil to Solution Ratio

2.5.3 Total concentration in plant

Dried plant tissues were crushed to a fine powder (motor and pestle) for elemental analysis. A known mass of plant tissue (0.4 g, dried) was combined with 1:2 (v:v) 65% HNO_3 : 30% H_2O_2, left overnight, then microwave-digested in a MARS 6 microwave digestion system for at 180^oC (15 min, ramp time and 15 min, holding time). Digests were diluted with high purity water to 2% acid content prior to analysis.

2.6 Element analysis

Digests (soils and plant material) and SEP extracts were analysed for As and Sb by Inductively Coupled Plasma-Mass Spectrometer (ICP-MS Agilent 7500CE or Thermo Scientific iCAP-Q). Analyses was performed using argon (plasma and carrier gas) with standard collision and reaction gas modes as per Price et al. (2013). Bi, Sc, Y, Rh and In were added to all samples (final concentration of 10 µg/L) as an internal standard to account for instrument drift. Blanks were carried throughout the digestion procedure and analysed every 15 samples. All soil and plant extracts were diluted to < 50 µg/L and rinsed with 2% HNO_3 between each sample analysis to remove any residue. Blanks and quality control standards (25 µg/L) were analysed at every 15 samples. Certified reference materials (CRM) were analysed to determine method recovery of As and Sb: standard reference material (SRM) 2711a-Montana II soil CRM for soils and SRM 1573a-tomato leaves for plant material (National Institute of Standards and Technology, Gatherburg, MD, USA).

There may be loss of As and Sb concentrations in plants during oven drying at 60 °C, but they are still comparable as all plant samples were dried using the same method. The low recovery of Sb in soils and plants relates to the difficulty in extracting Sb from environmental matrices (Mestrot et al. 2016). Therefore, care should be taken in comparing these results to other published studies.

2.7 Data analysis

All data are expressed as either means ± SD or ($n \leq 3$) or with 95% confidence intervals. Effect concentrations at the 10% and 50% inhibition levels (EC_{10} and EC_{50}, respectively) were calculated using the drc package (Ritz et al. 2016) in R (R Core Team 2017) by fitting a 3-parameter log-logistic model to concentration-response data, Equation 2.4 (Haanstra et al. 1985, Kader et al. 2016);

$$Y = \frac{a}{1 + e^{b(x-c)}}$$ Equation 2.4

Where Y is the tested parameters (length (cm) and dry mass (g) of root and shoot), a is the control measurement (cm/g), b is the slope factor, x is the logarithm value of concentration (mg/kg) and c is the logarithm EC_{50} value (mg/kg) (Kader et al. 2016).

These data and models were visualised using the ggplot2 package (Wickham 2016). Significant differences between treatments were evaluated using one-way ANOVA and Tukey's honest significant difference post-hoc test with an α value of 0.05. The linear regressions between total or SEP-bioavailable soil metalloid concentrations and accumulation in tissues were explored using R (R Core Team 2017).

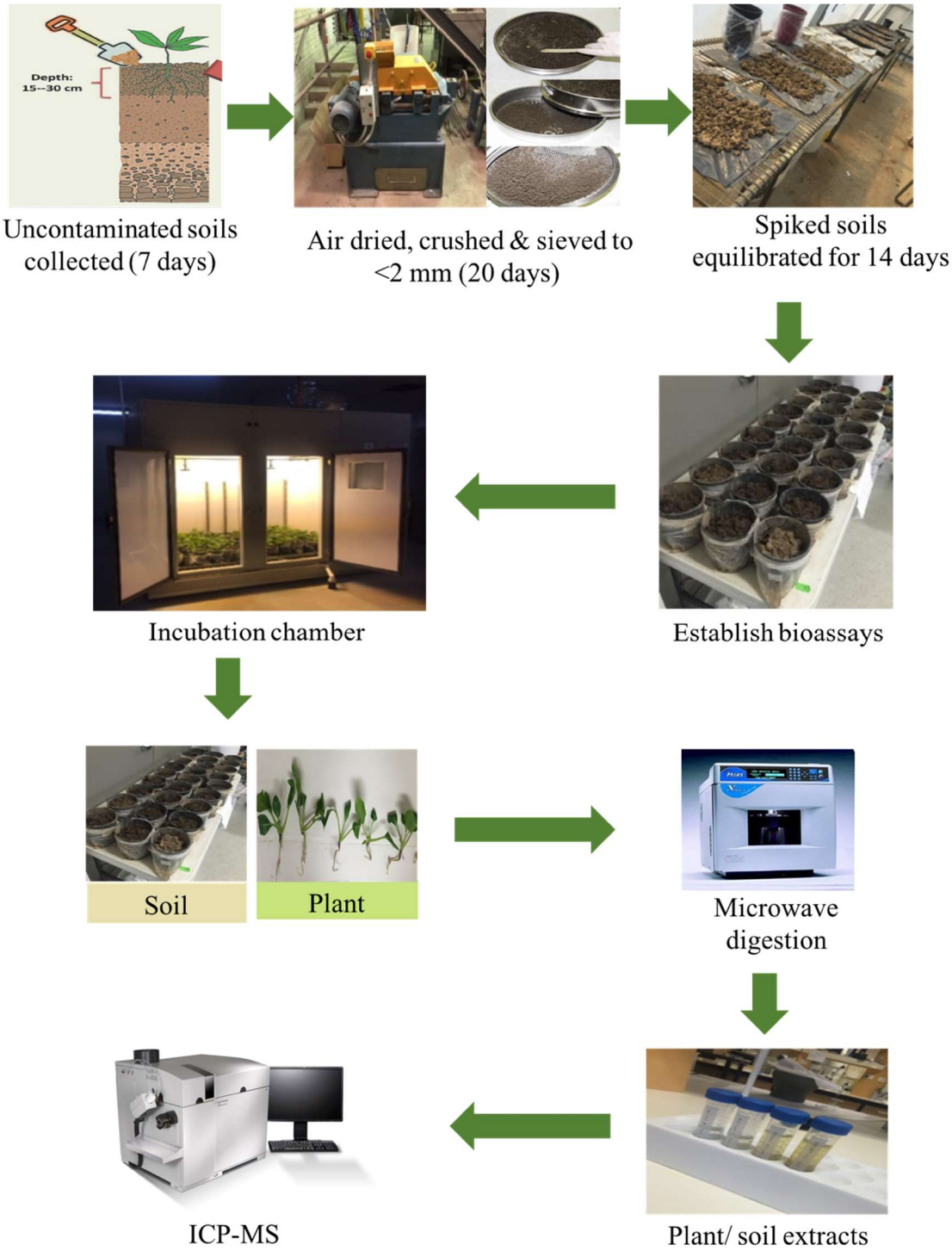
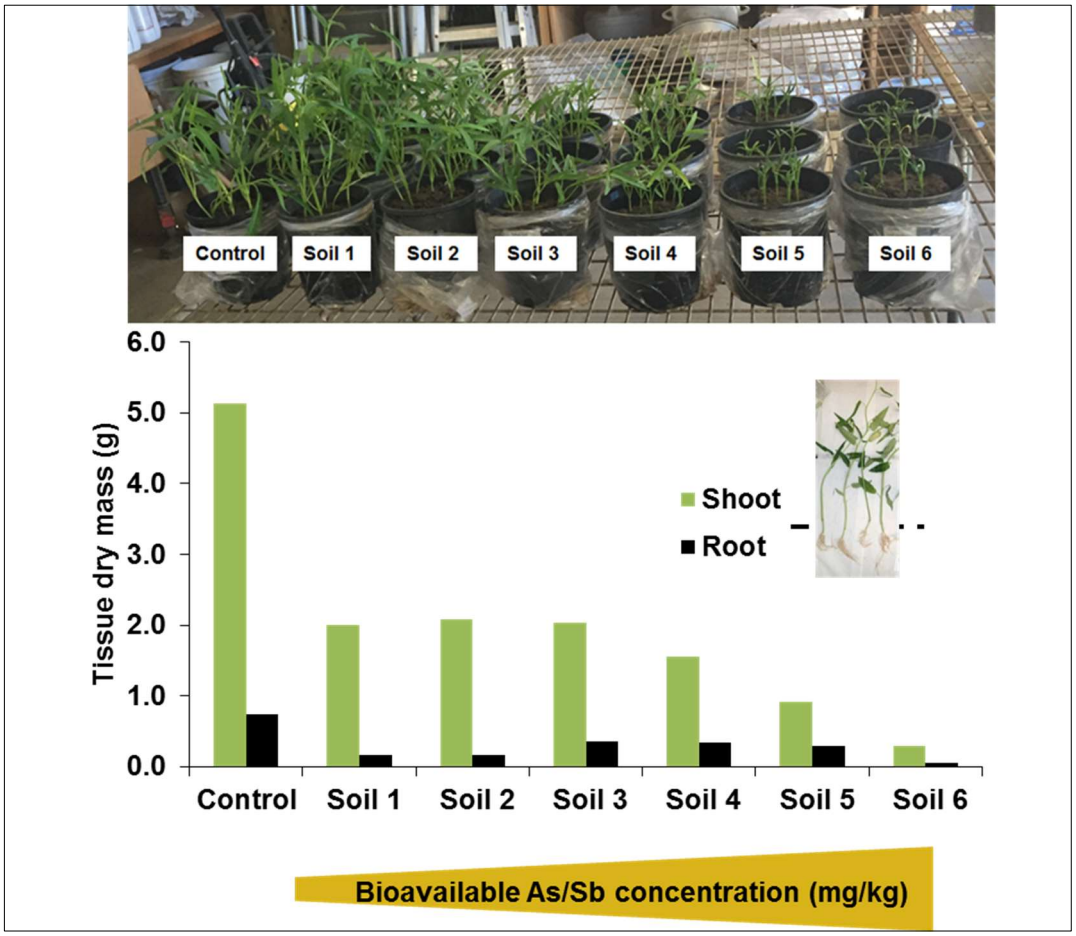


Figure 2.1. Summary of the method used in this study.

Chapter 3. Bioavailability and toxicity of As and Sb on *Ipomoea aquatica* grown in recently and historically co-contaminated soils



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LPE: Conceived the experiment design and carried out the experiment, Formal data analysis, prepared all figures and wrote the original manuscript draft. **GM and LKN**: contributed to the experimental design and editing the manuscript. **DFJ**: Supervision, provided resources and funds, contributed to review & editing of the manuscript.

3.1 Introduction

Soil is a major sink for As and Sb emitted by both natural and anthropogenic activities. In the soil, As and Sb may be present in free ionic forms, precipitated as solids, adsorbed on soil organic or inorganic constituents, exchangeable or structural constituents of minerals (primary and secondary) (Abbas et al. 2018). Metalloid concentrations in soils are measured using various operationally defined techniques. The 'total' concentration involves an aggressive digest that liberates metalloid ions bound to the crystalline structure of the minerals, bound in solid state organic matter and free ions and soluble organic and inorganic complexes in the soil solution (Alloway 2013). The metalloids present as free ions, soluble complexes and labile forms represent the mobile and bioavailable fractions in the soil (Alloway 2013).

The mobility and bioavailability of As and Sb in the soil is mainly controlled by adsorption-desorption reactions, chemical complexation with organic and inorganic ligands, biotic and abiotic redox reactions and precipitation-dissolution reactions (Section 1.3). The extent of these reactions is often influenced by environmental factors such as soil pH, type of sorbent, concentrations of competitive ligands, redox conditions and residence time (Wilson et al. 2010, Pigna et al. 2015, Liang et al. 2016). Among these, time is an important factor as the nature and strength of complexation reactions changes over time (Figure 1.3). Arsenic and Sb in soil solutions often decrease with increased metalloid-soil residence time as they undergo processes including surface precipitation/surface oxidation, Ostwald ripening, cavity entrapment, diffusion into microspores and/or incorporation into crystal lattices of the soils (Figure 1.3) (Naidu et al. 2008, Violante et al. 2008, Wang et al. 2015). Although the influence of these factors during soil ageing has been studied in individually contaminated soils, there is a lack of knowledge on co-contaminated soil. This is important in assessing their risks as many environments are contaminated with metalloids mixtures rather than one metalloid.

In some plants, As and Sb are found at concentrations <1 and <0.05 mg/kg (dry weight), respectively (Baroni et al. 2000, Abbas et al. 2018). The accumulation of higher concentrations would be toxic to the plant (Baroni et al. 2000, Abbas et al. 2018). However, some hyperaccumulator plants are able to accumulate high concentrations of As and Sb in their tissues without any toxicity (Martínez-Sánchez et al. 2011). Arsenic and Sb accumulated in plant roots translocate to shoots and leaves through xylem and

phloem (Section 1.4.2) (Bergqvist et al. 2014). While knowledge of As toxicity is well established for plants (Section 1.5), little is known about the toxicity of Sb which can also be readily accumulated by plant roots when present as soluble Sb(III) and Sb(V) in the soil solution (He et al. 1999). Antimony has been observed to induce moderate phytotoxicity to plants (Tisarum et al. 2015); however, the mechanisms of Sb phytotoxicity in plants have not been well studied. Further, the accumulation and toxicity of As and Sb in co-contaminated soil may be influenced by soil ageing and thus, more information is needed.

Plant bioassays allow inferences to be made about the potential toxicity of contaminants. Unlike chemical analysis, bioassays account for the physicochemical conditions that influence contaminant kinetics within the soil and assess the harmful effects on the plant. This research focuses on the agriculturally important water spinach (*Ipomoea aquatica*), a readily available tropical leafy vegetable common in many countries across the equator region (e.g., South East Asia), grown under aquatic or semi-aquatic conditions and known to be sensitive to soil metalloid contamination (Göthberg et al. 2002, Yang et al. 2012). On the other hand, As mobilized from sediments to ground water has been mainly observed in many regions of South East Asia. This chapter investigates the bioavailability, accumulation (in edible parts) and phytotoxicity of As and Sb on *I. aquatica* in both historically (aged) and recently contaminated soils.

3.2 Methods

3.2.1 Soil collection and preparation

Historically contaminated soil (denoted as HS) was collected from a decommissioned antimony processing facility in Urunga, NSW (Australia), a site with high recorded concentrations of 5850 mg As/kg and 7650 mg Sb/kg. The facility was operational from 1969 to 1974, owned and operated by Broken Hill Antimony Pty Ltd. For historically contaminated soils (aged for ~34 years), the bioassay concentration series (nominal Sb concentrations from 50-5000 mg/kg) were prepared by mixing the fields collected contaminated and control soils at various ratios using a cement mixer. Recently contaminated soils (aged for 14 days) (denoted as RS) were prepared by spiking the control soils with As (sodium arsenate) and Sb (potassium hexahydroxoantimonate) solutions to achieve As and Sb combined contaminated soil with nominal concentrations

from 40-1500 mg Sb/kg and 40-400 mg As/kg as described in Section 2.2.

3.2.2 *Ipomoea aquatica* bioassay

I. aquatica bioassays were established as described in Section 2.3.1. After 35 days, bioassays were terminated. Thirty-five days was chosen for termination as *I. aquatica* usually takes approximately 4-6 weeks to mature. The toxicity end points (shoot and root length and dry mass) were measured as per Section 2.3.2 and plant tissue was collected for As and Sb analysis.

3.2.3 Photosynthetic efficiency analysis

Photosynthetic efficiency of the plants was assessed on day 30 using a pulse-amplitude modulated (MINI-PAM) fluorimeter (Heinz Walz GmbH, Effeltrich, Germany). Plants were dark-adapted for 20 min before analysis. Values were reported as F_v/F_m, the ratio of the maximum fluorescence produced by a saturating flash (F_m) and the difference between fluorescence after dark adaptation (F_0) and a saturating flash (F_m) ($F_v = F_m–F_0$) (Nydahl et al. 2015). This ratio is a measure of the optimal maximum quantum photosynthetic efficiency.

3.2.4 Soil and plant analysis

The physical and chemical properties; soil pH, particle size, soil moisture, TOC, TKN and extractable phosphorus of test soils were measured as described in Section 2.4.

The sequential extraction procedure (SEP) was derived from Wenzel et al. (2001) as outlined in Section 2.5.1 and Table 2.1. The total As and Sb concentrations in soil and plant samples were extracted as per methods described in Sections 2.5.2 and 2.5.3, respectively.

All digests and SEP-extracts were analysed by ICP-MS Agilent 7500CE as described in Section 2.6. Method detection limits were 0.083 mg As/kg and 0.090 mg Sb/kg. Soils CRM recoveries were within 102% and 70% (n=3) of expected values for As and Sb respectively, and recoveries for plant CRM were within 107% and 99% (n=3) of expected values for As and Sb, respectively.

3.2.5 Data analysis

All data are presented as mean ± SD ($n \leq 3$) (3 pots per concentration, 6 plants per pot)

and were performed as per Section 2.7. The influence of bioavailable As and Sb fractions on plant growth were assessed by Pearson's *r* correlation. The EC_{10} and EC_{50} were calculated using JMP (Pro 11) using Equation 2.4.

3.3 Results and discussion

3.3.1 Soil characteristics

All test soils were fully characterised (Table 3.1) as silty-sand with 56-67% silt and 28-37% sand. For historically contaminated soils, a greater variability in soil characteristics was observed (compared to recently contaminated) because of the large ratios between highly contaminated and control soils required to achieve test concentrations (Section 2.2). In this research, the SEP-method does not capture the As and Sb bound to TOC in the bioavailable extracts, with the TOC-bound contaminants being accounted for in the residual fraction. This means that the 'bioavailable As and Sb' fractions are unaffected by variable TOC (3-8.6%). The soil moisture is crucial in determining the mobility of As and Sb in test soils, so was maintained at a maximum water holding capacity ~ 60% throughout the bioassays.

Table 3.1. Physical and chemical properties of the soils used in plant bioassays.

Characteristics	Historically contaminated soils (HS)	Recently contaminated soils (RS)
Sand > 62.5 µm – 2 mm (%)	22-63	30-33
Silt > 25-62.5 µm (%)	33-70	60-62
Clay 3.9-25 µm (%)	3.4-8.5	7.5-8.1
pH	4-7	5.5-6
Total organic carbon (%)	3.2-8.1	8.5-8.6
Bioavailable phosphorous (mg P/kg)	11-150	17
Total kjeldahl nitrogen (mg N/kg) ± SD	200 ± 160	570 ± 20

3.3.2 Bioavailability of As and Sb in soils

Analysis of total soil As and Sb concentrations showed that actual concentrations were generally in good agreement with the nominal values for all test soils (Table A2.1).The SEP-bioavailable As and Sb in soils included non-specifically bound (SO_4^{2-} extractable) and specifically bound (PO_4^{3-} extractable) metalloid fractions whereas SEP-total extractable fractions included the sum of all four SEP-extracts (Wenzel et al. 2001, Filella 2011). These will be referred to as SEP-bioavailable and SEP-total metalloid, respectively. For both As and Sb, SEP-total concentrations in historically and recently co-contaminated soils were significantly higher than the bioavailable concentrations (Table 3.2) and were proportional to the total soil concentration.

Greater metalloid lability was observed in recently co-contaminated soil, ranging from 20-40% for As and 19-65% for Sb; whereas in historically co-contaminated soil, the bioavailability ranged from 5-9.8 % for As and 0.5-1.4 % for Sb. The decreased metalloid bioavailability in aged soils is due to retention mechanisms such as adsorption, precipitation and co-precipitation (Juhasz et al. 2008, Wilson et al. 2010, Wang et al. 2015, Liang et al. 2016). For example, adsorption on to Fe, Al and Mn (hydr)oxides in soils is considered as the principal factor influencing the retention of As and Sb (Juhasz et al. 2008, Wilson et al. 2010). This is supported by Hammel et al. (2000) who found higher mobility of Sb (7.8-8.9%) in recently co-contaminated soils (aged for 6 months) than in soils from mining areas (0.02-0.21%).

Overall, in both treatments, the bioavailable Sb was much lower than bioavailable As except at the highest concentration (RS6) of recently co-contaminated soils (Table 3.2). This is shown in Figure 3.1, where bioavailable As dramatically increased with total concentrations in both historically and recently co-contaminated soils, while bioavailable Sb slightly increased in historically co-contaminated soils. The gradients (m) of these relationships showed that bioavailable and total soil concentrations for As ($m_{\text{As-}HS} = 0.05$) were 10 times greater than Sb ($m_{\text{Sb-}HS} = 0.005$) in the historically co-contaminated soils. In recently co-contaminated soils, the difference was not as great, with bioavailable Sb ($m_{\text{Sb-}RS} = 0.7$) only 1.6 times greater than bioavailable As ($m_{\text{As-}RS} = 0.4$) which may be due to the highest concentration (RS6). These results were supported by Ngo et al. (2016) who showed that Sb ($0.93 \pm 0.04\%$) had less bioavailability to radish than As ($5.7 \pm 0.2\%$) in historically co-contaminated soils after dilution.

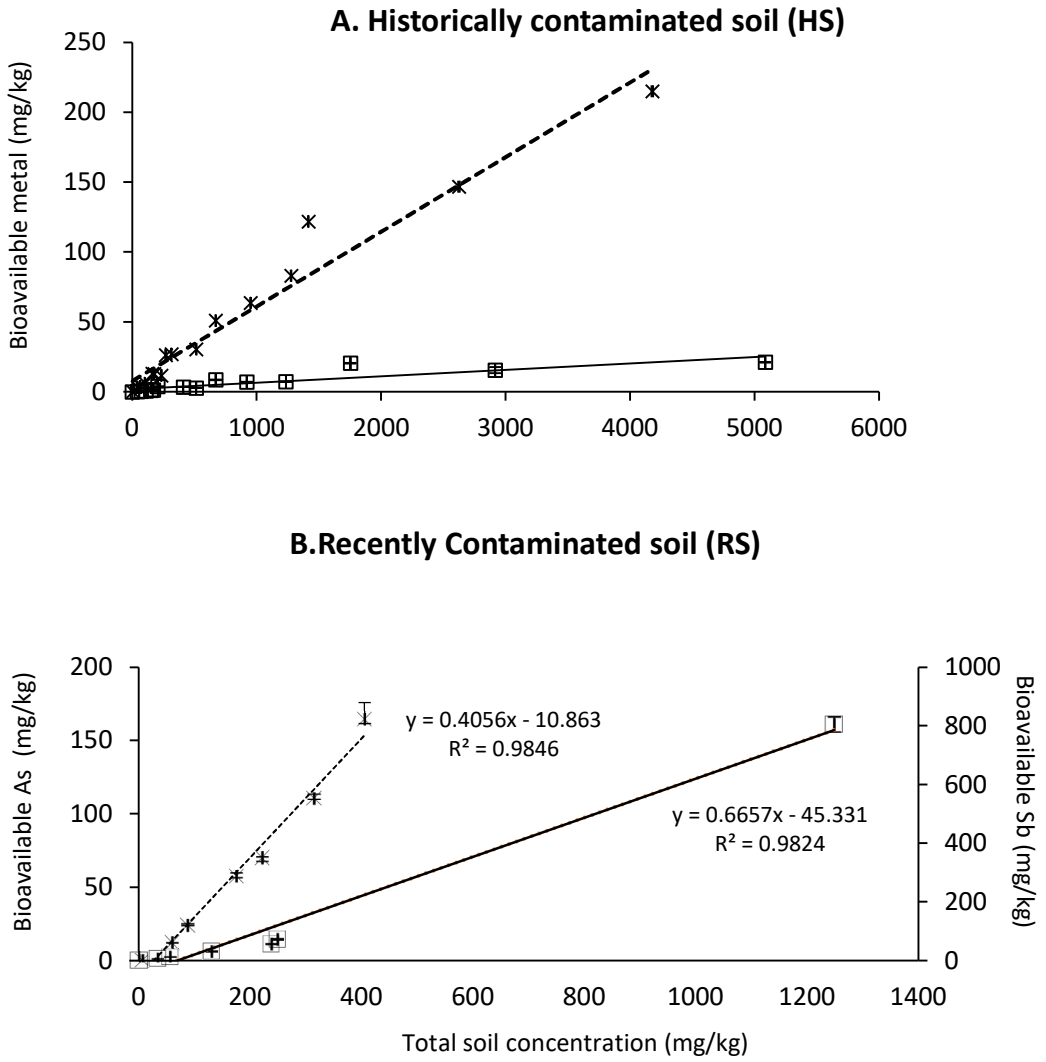


Figure 3.1. The relationship between total soil and SEP-bioavailable As (×, dashed line) and Sb (□, solid line) in bioassay soils (mg/kg) (mean ± SD, n ≤ 3).

Table 3.2. Total soil and bioavailable concentrations obtained by sequential extraction procedure (SEP) in historically (HS) and recently (RS) co-contaminated soils (mean ± SD, n ≤ 3). The percentage of the total As and Sb in the bioavailable fraction is also reported.

	Total soil metalloid		SEP-total metalloid		Bioavailable metalloid (extract 1 and 2)		% Bioavailability of total soil metalloid	
	As (mg/kg)	Sb (mg/kg)	As (mg/kg)	Sb (mg/kg)	As (mg/kg)	Sb (mg/kg)	As %	Sb%
Historically co-contaminated soils (HS)								
Control	5.4 ± 0.6	0.23 ± 0.02	5 ± 2	0.37 ± 0.09	0.2 ± 0.1	<0.09	5 ± 2	*
HS1	42.2 ± 0.3	40 ± 8	40 ± 3	25 ± 2	3.48 ± 0.07	0.310 ± 0.008	8.3 ± 0.2	0.8 ± 0.2
HS2	71 ± 4	70 ± 1	70 ± 15	45 ±15	4.13 ± 0.06	0.39 ± 0.02	6 .3 ± 0.4	0.55 ± 0.02
HS3	100 ± 15	100 ± 10	100 ± 5	65 ± 1	6.5 ± 0.2	0.56 ± 0.02	6.5 ± 0.9	0.51 ± 0.03
HS4	160 ± 50	160 ± 30	145 ± 20	95 ± 16	13.0 ± 0.1	1.69 ± 0.02	8.5 ± 2.3	1.1 ± 0.2
HS5	235 ± 60	150 ± 50	150 ± 5	93 ± 3	11.7 ± 0.4	1.07 ± 0.04	5.3 ± 1.6	0.8 ± 0.3
HS6	270 ± 15	210 ± 80	300 ± 5	195 ± 6	26.4 ± 0.3	3.78 ± 0.06	9.8 ± 0.5	2 ± 1
HS7	315 ± 35	410 ± 240	415 ± 90	240 ± 40	25 ± 2	2.8 ± 0.1	8 ± 1	0.8 ± 0.4
HS8	520 ± 50	510 ± 100	445 ± 15	265 ± 20	30.5 ± 0.6	2.78 ± 0.02	6.0 ± 0.6	0.6 ± 0.1
HS9	670 ± 215	670 ± 230	640 ± 25	420 ± 50	51 ± 1	8.6 ± 0.1	8 ± 2	1.4 ± 0.4
HS10	950 ± 90	900 ± 80	930 ± 10	570 ± 40	65 ± 1	7.1 ± 0.1	6.7 ± 0.6	0.8 ± 0.1
HS11	1280 ± 70	1230 ± 80	1150 ± 40	830 ± 50	80 ± 5	7.3 ± 0.2	6.5 ± 0.3	0.59 ± 0.04
HS12	1410 ± 90	1750 ± 170	1460 ± 30	1125 ± 40	120 ± 15	20.6 ± 0.2	8.6 ± 0.6	1.2 ± 0.1
HS13	2630 ± 150	2920 ± 240	2500 ± 100	1910 ± 5	143 ± 1	14.4 ± 0.1	5.4 ± 0.3	0.5 ± 0.1
HS14	4200 ± 200	5085 ± 500	3800 ± 200	3200 ± 400	210 ± 5	21.3 ± 0.3	5.2 ±0.1	0.42 ± 0.04
Recently co-contaminated soils (RS)								
Control	6 ± 0.3	0.3 ± 0.03	2 ± 1	0.31 ± 0.02	<0.08	<0.09	*	*
RS1	60 ± 10	35 ± 5	40 ± 1	42 ± 1	12.1 ± 0.2	6.40 ± 0.10	20 ± 3	19 ± 3
RS2	88 ± 3	56 ± 10	70 ± 2	80 ± 3	24 ± 1.0	13.0 ± 0.3	30 ± 2	23 ± 4
RS3	175 ± 2	130 ± 7	150 ± 5	170 ± 2	58 ± 2	31 ± 2	33 ± 1	23.9 ± 0.7
RS4	220 ± 7	240 ± 5	180 ± 1	282 ± 1	70 ± 0.6	58 ± 1	32 ± 1	24.2 ± 0.7
RS5	315 ± 5	250 ± 10	275 ± 3	332 ± 1	110 ± 3	74 ± 2	35 ± 1	30 ± 2
RS6	405 ± 4	1250 ± 30	380 ± 10	1850 ± 40	165 ± 10	800 ± 20	40 ± 3	65 ± 4

*Below limits of detection for bioavailable fractions

This study showed that ageing of contaminated soils decreased the bioavailability of As and Sb in co-contaminated soils. The bioavailability of As and Sb depends on residence time, soil properties and the total soil metalloid concentration. Due to the high residence time (~ 34 years) in historically contaminated soils, the percentage of As and Sb in soils that was bioavailable was < 10% and < 2%, respectively. However, in recently contaminated soils (aged for 14 days), the percentage of bioavailable As and Sb was < 40% and < 65%, respectively, even at low total soil concentrations (Table 3.2). In general, soil physical properties do not reveal the age of contaminated soils and thus it is difficult to describe the extent of metal lability using these parameters.

This research shows that bioavailability of As and Sb decreased with increasing residence time in the soil, even at similar total soil concentrations. For example, at a total soil concentration of 220 mg As/kg in HS6 and RS4 (Table 3.2), recently contaminated soil had a significantly higher bioavailable fraction (70 ± 0.6 mg/kg) compared to historically contaminated soil (11.7 ± 0.4 mg/kg). A similar pattern was observed for total Sb soil concentrations at approx. 130-150 mg Sb/kg, recently contaminated soil had a higher bioavailable fraction (31 ± 2 mg/kg) compared to historically contaminated soil (1.07 ± 0.04 mg/kg). The difference in sensitivity to As and Sb in *I. aquatica* would not have been observed if the dose-response analyses had only been interpreted using total soil concentrations. Prediction of labile fraction is important as this fraction of the soil can easily move to plant roots, increasing exposure to the contaminant, and effecting plant growth. Therefore, measurement of the bioavailable fraction of As and Sb is important to predict the plant toxicity relative to the age of contaminated soils.

3.3.3 The effect of As and Sb on the growth of *Ipomoea aquatica*

Three endpoints were used to assess the phytotoxicity of As and Sb on *I. aquatica*: biomass (roots and shoots), length (roots and shoots) and photosynthetic efficiency of foliage. Each endpoint was considered in the context of total and SEP-bioavailable metalloid concentrations in both historically and recently co-contaminated soils.

Effects on biomass

The *I. aquatica* successfully germinated on all test soils (Figure 3.2). Following 35-days, the moisture of roots and shoots in both soil treatments (historically and recently co-contaminated) was consistently between 60-90% (Table A2.2). There was no evidence that dehydration contributed to the adverse effects observed in plant physiology during

the bioassays. However, there was a significant dose-dependent decrease in tissue dry mass when compared to both total and SEP-bioavailable metalloids concentrations in historically and recently co-contaminated soils (Figure 3.3 and Figure A2.1, respectively). Effective concentrations at 10% and 50% inhibitions (EC_{10} and $EC_{50,}$ respectively) for tissue dry mass were derived as per Equation 2.4 for both total soil and SEP-bioavailable concentrations (Table 3.3). In both historically and recently co-contaminated soils, there was a decrease of 98-99% in root and shoot dry mass (Figure 3.3). However, only shoot followed a good relationship with the logistic response model, with respect to both total and SEP-bioavailable concentrations in soils.

For historically co-contaminated soils, the EC_{10} and EC_{50} values showed that the SEP-bioavailable Sb was more toxic than As to both root and shoot dry mass while no significant differences ($EC_{50\text{-}As}$: 11(4-30) and $EC_{50\text{-}Sb}$: 2(0.14-33)) were observed in recently co-contaminated soils (Table 3.3). Further, the lower EC values in historically co-contaminated soils showed that shoot drymass was more affected than root drymass in regard to both As and Sb. Similarly, the root and shoot dry mass of wheat exposed to As in a 21 d bioassay also showed that shoots were more sensitive than roots (Mojsilovic 2009). However, this appears to be species specific for Sb, because root growth was found to be more sensitive than shoot growth in Chinese cabbage seedlings, wheat, cucumber and mung bean exposed to soil Sb for 5 days (Baek et al. 2014). Little information is available on the combined toxicity of As and Sb relative to root and shoot dry mass.

In general, when roots are exposed to As, root length and proliferation is inhibited; once metalloids translocate to the shoots, these metalloids can inhibit plant growth by slowing or arresting growth and biomass accumulation (Ngo et al. 2016). At sufficiently high concentrations, As interferes with critical metabolic processes (Finnegan et al. 2012), which can lead to plant death, which was consistent with chapter 3 of this thesis. Although recent studies showed potential uptake of Sb by plants, its toxicity is still unclear. Toxic effects observed in plants include increased accumulation of lipid peroxidative products and enhanced oxidative stress in roots of maize plants (Vaculíková et al. 2014) and reduced uptake of essential elements leading to reduced plant growth (Feng et al. 2013).

Shoot dry mass exhibited more sensitivity to SEP-bioavailable concentrations than total soil metalloid concentrations. This is expected because the bioavailable component is a fraction of the total soil concentration which is readily available for plant uptake. In

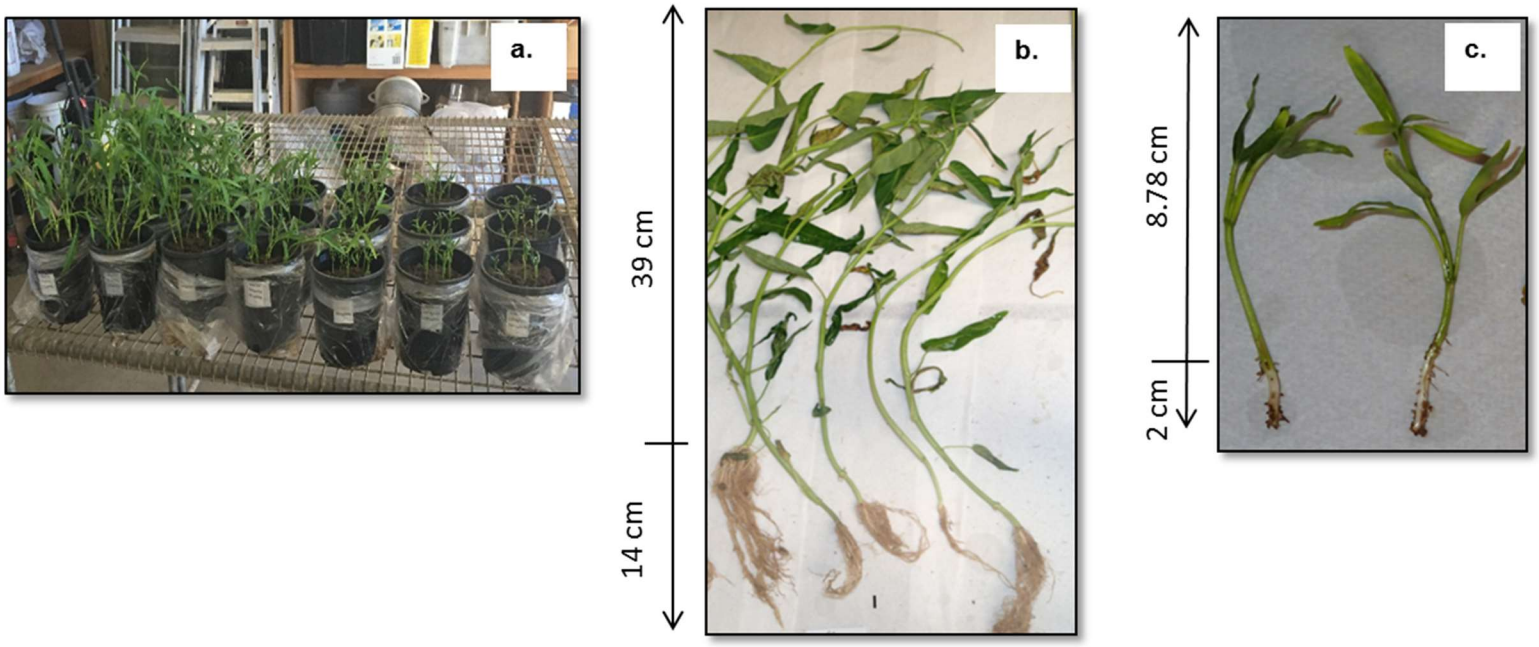


Figure 3.2. The bioassay of *Ipomoea aquatica* in recently co-contaminated soil a). series of plants exposed to As and Sb co-contaminated soil on day 14 (exposure concentrations increase from left to right), b). Control on day 35, and c). HS14 on day 35.

Table 3.3. EC_{10} and EC_{50} estimates for tissue mass of *Ipomoea aquatica* (35-day bioassay) exposed to As and Sb in historically and recently contaminated soils. For recently contaminated soils, there was a poor relationship between root length and exposure concentration, therefore only the EC_{50} for shoot elongation were estimated.

			$EC_{10\text{-}As}$ mg/kg (95% CI)	**$EC_{10\text{-}Sb}$ mg/kg (95% CI)**	**$EC_{50\text{-}As}$ mg/kg (95% CI)**	**$EC_{50\text{-}Sb}$ mg/kg (95% CI)**
Total soil concentration						
Historically contaminated soil	Dry mass	Roots	15 (12-637)	73 (9-580)	1263 (816-956)	1360 (864-2141)
		Shoots	69 (30-156)	61 (23-160)	684 (530-884)	703 (518-954)
	Length	Roots	1344 (1130-1590)	306 (57-1640)	1463 (1292-1657)	2381 (1350-4180)
		Shoots	75 (19-294)	60 (14-262)	1491 (1050-2120)	1630 (1122-2370)
Recently contaminated soils	Dry mass	Shoots	- [a]	- [a]	52 (17-160)	13 (1-160)
	Length	Shoots	- [a]	- [a]	236 (145-385)	286 (142-579)
Bioavailable soil concentration						
Historically contaminated soil	Dry mass	Roots	9 (2-46)	0.7 (0.05-8)	83 (53-120)	9 (5-16)
		Shoots	4 (1-12)	0.5 (0.13-2)	49 (37-65)	5 (3-8)
	Length	Roots	5 (8-121)	8 (6-11)	133 (91-194)	13 (3-35)
		Shoots	4 (0.6-31)	0.5 (0.039-6.37)	96 (64-145)	12 (7-19)
Recently contaminated soils	Dry mass	Shoots	- [a]	- [a]	11 (4-30)	2 (0.14-33)
	Length	Shoots	- [a]	- [a]	80 (45-140)	96 (42-219)

[a] Derived EC values were lower than the lowest test concentration (<5 mg As/kg, <0.2 mg Sb/kg), or unable to be determined.

addition, the total soil concentrations depicted equivalent toxicity from As and Sb, with the EC_{50} for shoot biomass at 684 (530-884) mg /kg and 703 (518-954) mg/kg, respectively (Figure 3.3 and Table 3.3), which were not significantly different. However, EC_{50} for SEP-bioavailable concentrations showed that there was a clear difference between As (49 (37-65) mg/kg) and Sb (5 (3-8) mg/kg) toxicity for shoot dry mass. Therefore, evaluation of SEP-bioavailable EC_{50} values is important in field studies to identify metalloid toxicity on plant growth. The findings of this research have also been supported by multiple studies, Liu et al. (2012) demonstrated that when wheat was exposed to high levels of As (≥ 80 mg/kg) in soils spiked with Na_2HAsO_4 and equilibrated for 10 d, there was a significant decrease in the biomass from 89 ± 8 to 61 ± 4 g. Baek et al. (2014) reported that the EC_{50} values for roots and shoots exposed to Sb in soil for 5 days were in the range of 798-900 mg/kg for mung bean, 850-1171 mg/kg for Chinese cabbage, and 1075-1126 mg/kg for cucumber. Also, the sensitivity of total soil As was a poor indicator of phytotoxicity to green beans, lima beans, spinach and tomato plants relative to bioavailable As (Mojsilovic 2009).

Effects on length

For both historically and recently co-contaminated soils, strong correlations ($r^2 > 0.85$) were observed for both root and shoot lengths with increasing total soil and SEP-bioavailable concentrations (Figure 3.4 and Figure A2.2, respectively). Once again there was a poor dose-response relationship between root length and exposure concentrations in recently co-contaminated soil, therefore only the EC_{50} for shoot length was estimated. The total soil metalloid, EC_{50} in HS soil occurred at 1491 mg As/kg and 1630 mg Sb/kg, with much lower EC_{50} values obtained for SEP-bioavailable concentrations (96 mg As/kg and 12 mg Sb/kg). In recently co-contaminated soil, the estimated EC_{50} values for shoot length were 236 mg As/kg and 286 mg Sb/kg of total soil and 80 mg As/kg and 96 mg Sb/kg of SEP-bioavailable concentrations. This implies that shoot lengths were more sensitive (much lower EC_{50}) to recently co-contaminated compared to historically co-contaminated soils (Table 3.3). In addition, total soil concentrations were a less indicative measure of plant sensitivity compared to SEP-bioavailable concentration in both soils, which was consistent with plant tissue dry mass inhibition (Section 3.3.3). Interestingly, when comparing EC_{50} values of shoot length (Table 3.3) in recently co-contaminated soils, the toxicity of SEP-bioavailable As was greater than Sb but considering the CI at 95% level this observation is insignificant.

Overall, plants were less effected (dry mass and shoot length) by aged As and Sb co-contaminated soils, ~34 years after mining. Similar results were reported by Song et al. (2006) using barley root elongation, with total soil As EC_{50} between 2.0 to 3.5 times higher after 3 months of ageing, with the bioavailable fraction (defined as SEP non-specifically and specifically bound As) of the soil decreasing over the time. Although Sb phytotoxicity has been observed in some studies, EC_{50} for plant growth is not well established.

The similar shapes of dose-response curves between total soil and SEP-bioavailable metalloid concentrations were observed in historically co-contaminated soils (Figure 3.3 and Figure 3.4). For total soil concentrations, the dose-response curves for As and Sb overlapped and showed similar EC_{50} values. However, a clear separation was observed between As and Sb dose-response curves using the SEP-bioavailable concentrations, and the greater sensitivity for Sb was reflected by different EC_{50} values for SEP-bioavailable As and Sb. Song et al. (2006) also reported that barley root elongation in 16 different soils spiked with As had EC_{50} ranging from 27 to 458 mg/kg whereas a much narrower range of EC_{50} values was obtained based on the bioavailable fraction. The EC_{50} obtained for shoot dry mass and length showed SEP-bioavailable Sb was more toxic than As in the aged soils. To support these results, Oorts et al. (2008) observed 10% inhibition in plant growth (root elongation of barley and shoot biomass of lettuce) corresponded to total soil concentrations of 510 mg/kg in the soil equilibrated with Sb_2O_3 for 31 weeks whereas no toxicity was observed one week after soil spiking. However, in the same study, clear toxicity was observed in the soil amended with $SbCl_3$ after one week of equilibration, possibly due to the decrease in pH and increase in salinity. Thus, it is evident that the toxicity of Sb is influenced by the chemical form and speciation in the soils (Feng et al. 2013), and that this warrants further investigation.

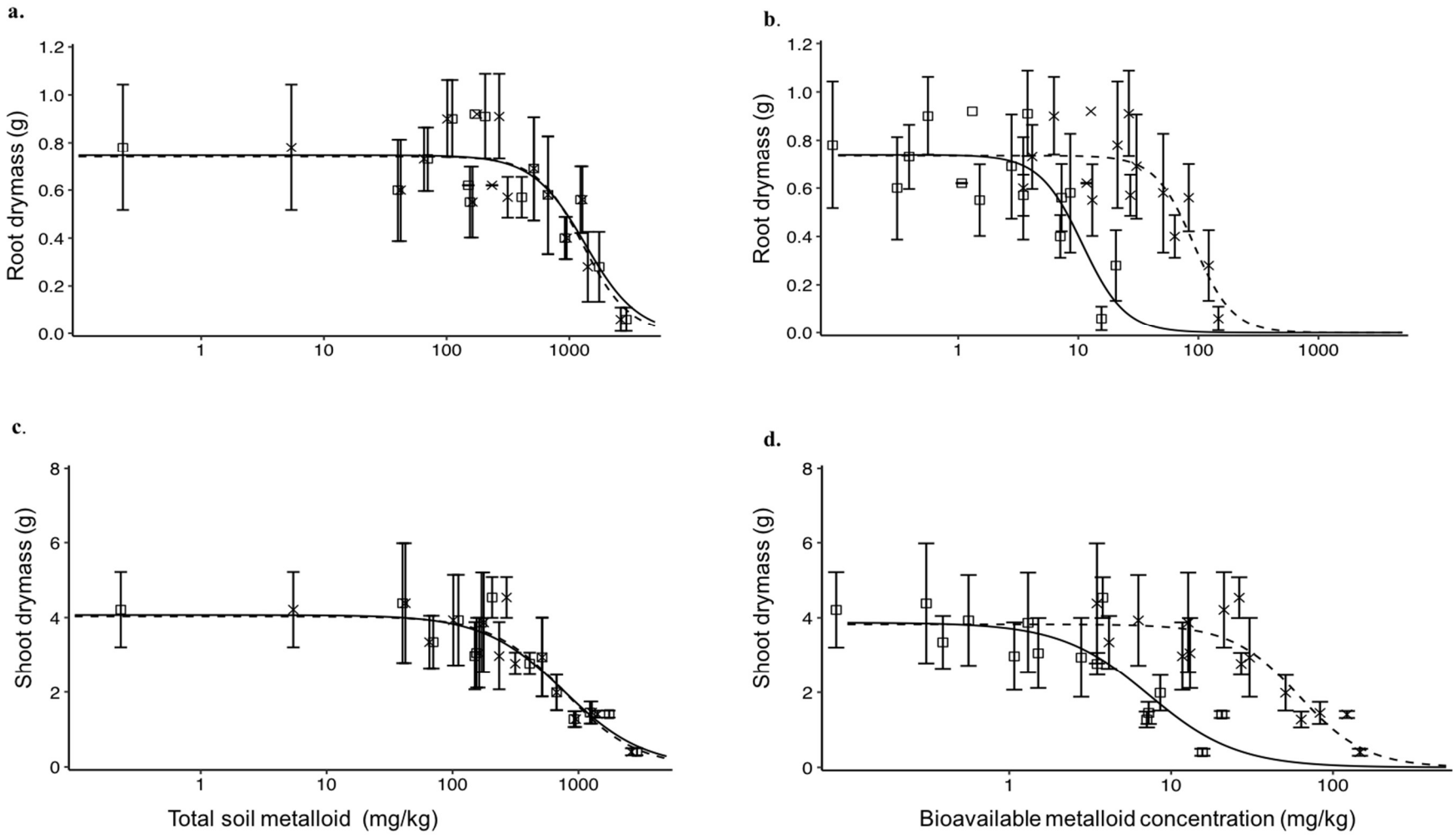


Figure 3.3. The relationship between *Ipomoea aquatica* root and shoot dry mass with (a, c) total soil and (b, d) SEP-bioavailable concentrations in historically co-contaminated bioassay soils. As (×, dashed line) and Sb (□, solid line). Shoot and root dry mass reported as mean ± SD, n ≤ 3.

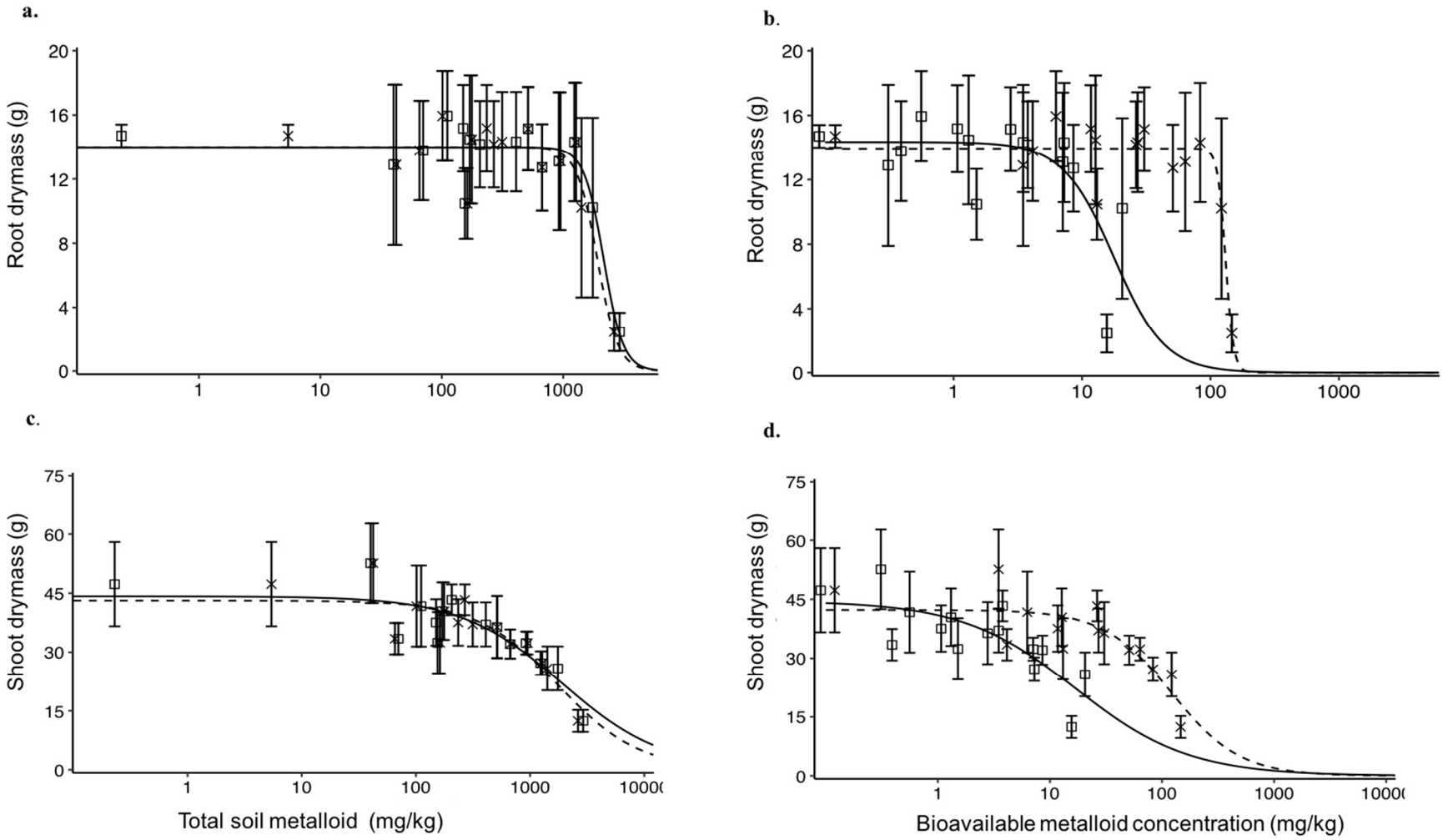


Figure 3.4. The relationship between *Ipomoea aquatica* root and shoot length with (a, c) total soil and (b, d) SEP-bioavailable concentrations in historically co-contaminated bioassay soils. As (×, dashed line) and Sb (□, solid line). Shoot and root lengths reported as mean ± SD, n ≤ 3.

3.3.4 Effects on the photosynthetic efficiency of leaves

Chlorophyll fluorescence ratio (Fv/Fm) measured the efficiency of plant leaves in utilising light for photosynthesis (Bjorkman et al. 1987). In general, under optimal conditions most plants have an Fv/Fm ratio of ~ 0.8, and in the presence of plant stress, the maximum chlorophyll fluorescence increases, decreasing the Fv/Fm ratio. Interestingly, the average Fv/Fm ratio across all test soils were 0.83 ± 0.012 and 0.83 ± 0.019 in historically and recently contaminated soils, respectively (Figure A2.3). Exposure of *I. aquatica* to As and Sb had no effect on the chlorophyll fluorescence signals and hence photosynthetic efficiency. However, these results are in contrast to Feng et al. (2013) who stated that Sb has the ability to affect photosynthesis in plants by inhibiting chlorophyll synthesis and maximum photochemical efficiency (Fv/Fm). Pan et al. (2011) also showed that chlorophyll fluorescence in the leaves of 3 week old seedling had decreased Fv/Fm from 0.82 at 50 mg kg^{-1} Sb to 0.62 at 1,000 mg kg^{-1} Sb, indicating that photosystem II was a target site for Sb toxicity. Sb was also shown to inhibit PSII in *Synechocystis* sp. (Zhang et al. (2010) following exposure to 1.0–10.0 mg L^{-1} Sb(III) by disturbing the electron transport from QA^- to QB/QB^-, increasing the flux of dissipated energy and decreasing the index of the maximum quantum yield (Feng et al. 2013). It has been reported that different forms of Sb had different effects on plant growth and therefore, the understanding of the speciation of Sb in the soils is important. The soils used in these experiments contain Sb(V) and no effects on Fv/Fm in water spinach were observed; however, the test soils used in this research were co-contaminated with As, and this may have had an antagonistic effect.

3.3.5 As and Sb accumulation in the edible parts of *Ipomoea aquatica*

I. aquatica contains a high level of nutrition, making it an important agricultural species (Marcussen et al. 2008). Following 35-days of exposure, the accumulation of As and Sb in *I. aquatica* showed different relationships (Figure 3.5) between historically and recently co-contaminated soils. In historically co-contaminated soils, As in shoots (edible part of plant) increased proportionally with total soil concentrations up to a maximum of 163 mg/kg in HS13 (total soil concentration; 2630 mg/kg and SEP-bioavailable concentration; 143 mg/kg) while the plants in HS14 were died. In recently co-contaminated soils the maximum tissue concentration was reached at much lower

concentrations, as As in shoots peaked at 78 mg/kg at 220 mg/kg total soil concentration and 70 mg/kg SEP-bioavailable concentration in soils then decreased at the top three concentrations (RS4-RS6) where soil bioavailability ranged from 40.3 to 45 % (Figure 3.5). These results showed that the accumulation of As in historically co-contaminated soils was dose dependent, consistently increasing with the exposure concentration, while recently co-contaminated soils showed a decrease in the accumulation of As at higher exposure concentrations due to a decrease in plant biomass (toxicity).

Excessive uptake of As is toxic to plants as it reacts with many enzymes and decoupling phosphorous transport in plant systems resulting in the inhibition of root growth and death (Kabata-Pendias et al. 2010). Under aerobic conditions, AsO_4^{3-} accumulates in cells via the phosphate transport system and forms unstable ADP-As (adenosine di-phosphate arsenate) which disrupt the energy production in plant tissue cells. However, the reduction of As(V) to As(III) readily occurs in plants, As(III) is highly toxic to plants because it reacts with sulfhydryl groups (-SH) in enzymes and proteins, causing them to lose their functionality (Meharg et al. 2002). In contrast Pigna et al. (2015) reported that As tolerance and detoxification in plants was associated with many mechanisms such as chelation, compartmentalization, biotransformation and cellular repair. For example, hyperaccumulator plants convert As(V) to As(III) which allows the plant to accumulate more As with no phytotoxicity due to the formation of less toxic As(III)-thiol complexes and As(III) phytochelatins (PC) (Pigna et al. 2015). Arsenic resistant plants can accumulate excessive amounts, with up to 3470 mg/kg in *Agrostis tenuis* and 560 mg/kg in *H. lanatus* (Meharg et al. 2002). Other plant species are less tolerant, for example, Soybean and cotton yields are affected when tissue arsenic exceeds 1 and 4 mg/kg, respectively, and barley tissue concentrations of 20 mg/kg inhibited growth.

Antimony concentrations in shoots reached 27 mg/kg at 1750 mg/kg total soil and 20.6 mg/kg SEP-bioavailable concentrations (HS13) in historically co-contaminated soils while in recently co-contaminated soils, Sb reached 100 ± 18 mg/kg at total and SEP-bioavailable concentrations of 1250 and 800 mg/kg, respectively (RS6) (Figure 3.5). Thus, Sb showed a proportional increase in tissue accumulation with the total soil and SEP-bioavailable concentrations in both HS and RS bioassay soils. However, little is known about Sb metabolism in cells due to variable data in plants. Kabata-Pendias et al. (2010) reported that generally in agricultural plants Sb concentrations range from <2 to

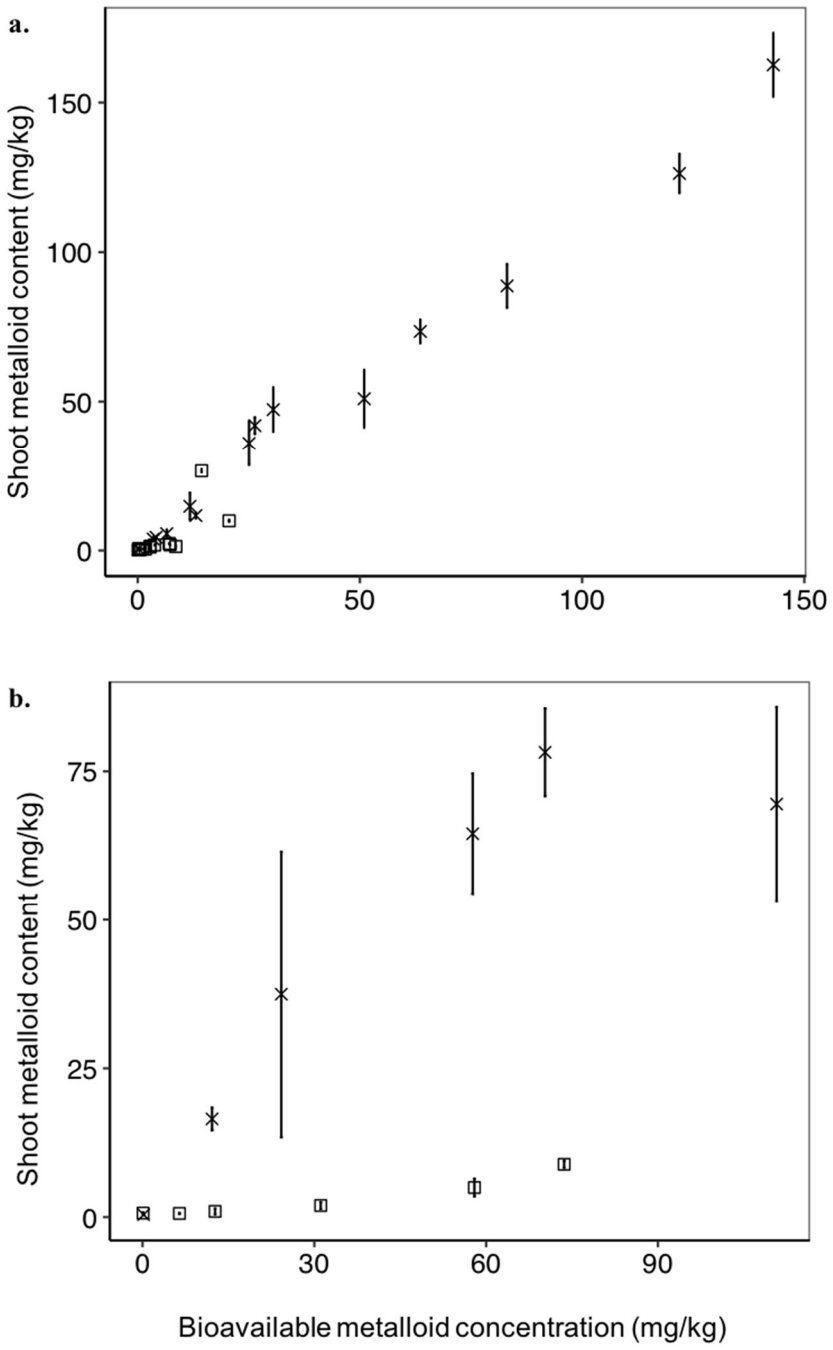


Figure 3.5. The accumulation of As (×) and Sb (□) in shoots (edible parts) of *Ipomoea aquatica* as SEP-bioavailable concentration increases (mg/kg) a). historically (Control and HS1-HS13) b). recently (Control and RS1-RS5) co-contaminated soils. Mean ± SD, n ≤3. (Table S1 for data)

29 µg/kg, with others reporting that Sb was specially located in leaves and shoots of vegetables and crop species whereas storage organs had lower concentration ranging from < 0.02 to 0.06 mg/kg (Hammel et al. 2000). This is consistent with a previous study on *R. sativus* by Ngo et al. (2016). Further to this, Hammel et al. (2000) also showed data for very high Sb concentrations from 0.15-1.13 mg /kg in spinach edible parts grown in total soil concentrations 18.6 to 266.3 mg Sb/kg compared to other vegetables and crop species. This suggests that water spinach can accumulate higher Sb concentrations from the contaminated soil in edible parts which is consistent with the results of this chapter. Interestingly, Hammel et al. (2000) also reported that vegetable crops grown in spiked contaminated soils (1000 mg/kg total Sb) have relatively high tissue Sb concentrations compared to historically contaminated mining sites. This is consistent with the results presented here which show that in recently co-contaminated soils *I. aquatica* shoots had greater concentration of Sb compared to that of historically co-contaminated soils. He (2007) reported 5-10 mg Sb/kg in plant tissues to be excessive or toxic for the plant growth. However, Feng et al. (2013) and He (2007) showed that Sb could reach 65.5 and 54 mg/kg with no observed phytotoxicity in rice and radish leaves, respectively. Similarly, Sb concentrations in shoots of *I. aquatica* used in this research has high concentration, but no toxicity on plant growth. Thus, Sb showed dose dependent accumulation within both bioassay soils, which resulted in impaired shoot growth but no physical distortions.

These results show that *I. aquatica* generally accumulated more As in shoots compared to Sb, but the relationship between ecological endpoints (shoot biomass and length) and SEP-bioavailable As and Sb showed that Sb has a greater impact on phytotoxicity. Thus, the toxic EC_{50} effect occurring at lower Sb concentrations compared to As could be due to the shared use of detoxification mechanisms. The main detoxification mechanisms of As and Sb in higher plants includes the reduction of As(V) and Sb(V) to As(III) and Sb(III), respectively followed by sequestration in vacuoles or in the xylem via silicic acid transporters (Garg et al. 2011, Zheng et al. 2012, Pigna et al. 2015). This mechanism helps to immobilise and isolate As and Sb in the plant. On the other hand, lipid peroxidation is considered as the most damaging process in plants under As and Sb stress. This produces malondialdehyde (MDA) which forms conjugates with both DNA and proteins in the plant cells while disrupting their action. So, the presence of both As and Sb can

enhance the production of MDA and cause more damage to the cells. However, it is the lack of physical deformities in this agriculturally important species that indicates *I. aquatica* grown in contaminated soils present a risk to human health via consumption. The consumption of vegetables (including leafy vegetables) is estimated to accounted for 26% (Sb) and 18% (As) of total daily intake (Wu et al. 2011). Wu et al. (2011) estimated that the total tolerable daily intake (TDI) is 360 µg Sb/day and 129 µg As/day. Marcussen et al. (2008) reported intakes based on a person with body weight of 46 kg who consumes 30 g (wet mass) *I. aquatica* per day containing 0.10-0.19 mg As /kg, which resulted in 5.7% As of the TDI. At the EC_{50} for shoot biomass inhibition, *I. aquatica* shoots accumulated 51-73 mg As/kg and 1.8-2.3 mg Sb/kg (dry mass) from historically contaminated soils. Based on shoot moisture of 90%, this is equivalent to 57-81 mg As/kg and 2-3 mg Sb/kg of wet mass.

If 30 g is ingested daily, this would be 1700-2400 µg As/day and 60-77 µgSb/day, which is above the 129 µg As/day TDI, but well below the 360 µg Sb/day (Wu et al. 2011). At the exposure concentration in HS13 soils, biomass was severely reduced yet the Sb in shoots reached 27 mg Sb/kg. Ingestion of 30 g shoots from these plants would result in 810 µg Sb/day, almost 2 times the TDI. Although these soil concentrations are very high, this still demonstrates a risk to human health.

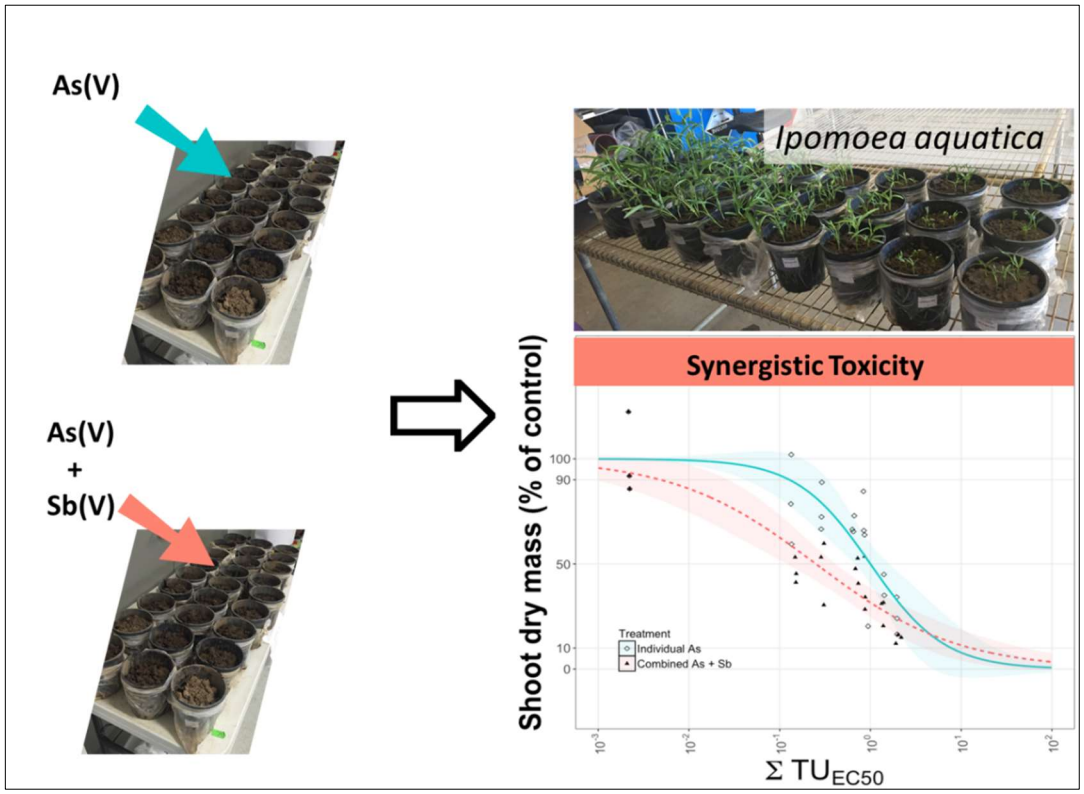
3.4 Conclusion

The Chapter 3 of this thesis investigated and compared the bioavailability of As and Sb in historically (aged for ~34 years) and recently (aged for ~ 14 days) co-contaminated soils and their toxicity on *I. aquatica*. The percentage bioavailability of As and Sb in recently co-contaminated soil was greater than in historically co-contaminated soils. The toxicity indicators showed that *I. aquatica* tissue dry mass and lengths were drastically decreased with increasing total and SEP-bioavailable As and Sb concentrations in soil, with no detectable effect in photosynthetic efficiency. Based on effective concentrations at 10% (EC_{10}) and 50% (EC_{50}) growth inhibition, recently co-contaminated soil was more toxic to *I. aquatica* growth than historically co-contaminated soil, with greater toxicity from Sb in both soils. The dietary exposure of As and Sb through *I. aquatica* may cause potential health risks to humans as it accumulates relatively higher concentrations without showing any serious impacts on its physical appearance.

Ageing and dilution of historically co-contaminated mining soils can reduce As and Sb

bioavailability. However, as As and Sb often co-occur in mining sites, their interactions could impact on the bioavailability and thereby, on accumulation and toxicity in plants. Therefore, the next chapter will investigate the impacts of ageing on the bioavailability, accumulation and toxicity of As and Sb.

Chapter 4. Interactive effects of As and Sb on bioavailability, accumulation and *Ipomoea aquatica* growth in co-contaminated soil



This chapter has been published.

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"**LPE**: Conceived the experiment design and carried out the experiment, Formal data analysis, prepared all tables and figures, wrote the original manuscript draft. **DK**: Supervision, contributed to the Statistical data analysis and preparing figures using R studio software, preparing editing the manuscript. **AH:** Supervision, contributed to the Statistical data analysis and editing the manuscript. **DFJ**: Supervision, provided resources and funds, contributed to review & editing of the manuscript.

4.1 Introduction

Arsenic (As) and Antimony (Sb) are metalloids that often co-occur in mineral deposits and instances of environmental pollution. Recent studies have shown that Sb concentrations have increased compared to As in contaminated soils due to anthropogenic activities such as mining, smelting, fossil fuel combustion and sewage sludge incineration (Fu et al. 2011, Ren et al. 2014, Cai et al. 2016). Contaminated soils can reach concentrations of As and Sb as high as 38600 (in mining areas) and 17500 (at a shooting range) mg/kg, respectively (Wilson et al. 2010). Furthermore, off-site migration can lead to As and Sb co-contamination in ground waters and agricultural lands near mining areas. This can lead to contamination often well above the maximum permissible metalloid content for agricultural soils of 5 mg Sb/kg and 20 mg As/kg (Wang et al. 2006, Casado et al. 2007, Feng et al. 2008, Bhattacharya et al. 2010).

Contaminant mixture studies are important because interactions between multiple contaminants can alter expected toxicity compared to single exposures (Wang et al. 2006, Bhattacharya et al. 2010). The toxicity of metalloid mixtures may be equal to the sum of the fractional toxic effects of individual components or higher/lower than the sum due to synergistic/antagonistic interactions, respectively (Wang et al. 2006, Li et al. 2014). For example, studies have shown that concentrations of contaminants below their 'no observed effect' concentrations may cause toxicity when present in mixtures (Wang et al. 2006, Okkenhaug et al. 2012, Barać et al. 2015). While As and Sb often co-occur in soils, limited information is available on competitive interactions of these metalloids to accumulation and toxic effects in plants (Ngo et al. 2016).

Arsenic and Sb have comparable speciation in soil and aqueous compartments because of similar oxidation states of III to V and frequently as tri- and pentavalent oxyanions. However, recent studies highlight that the different coordination structures of As(V) and Sb(V) lead to different geochemical behaviours under aerobic conditions (Tschan et al. 2009, Wilson et al. 2010, Fu et al. 2016). These differences may cause unexpected interactions to their toxicity and accumulation when present as a mixture. For example, antagonism from direct competition where the metalloids might compete for the same binding site or uptake pathway (Andrewes et al. 2000).

The toxicity and accumulation potential of As and Sb varies between plant species (Okkenhaug et al. 2012, Li et al. 2014, Ren et al. 2014). Radish, maize, potato, corn, rye,

wheat and coriander have all been shown to accumulate As or Sb in their edible parts to different extents from contaminated soils (Gulz et al. 2005, Barać et al. 2015, Zeng et al. 2015, Ngo et al. 2016). However, these results only reflect single-metalloid exposures, despite As and Sb often co-occurring in soils.

This chapter investigates the effects of As and Sb co-contamination on bioavailability, plant toxicity and accumulation in the commercially important agricultural plant, water spinach (*Ipomoea aquatica*). To do this, plants were exposed to As and Sb individually ($As_{(Individual)}$, $Sb_{(Individual)}$) and as a mixture ($As + Sb_{(Combined)}$) in a concentration series based on representative soil concentrations near Sb mine sites.

4.2 Methods

4.2.1 Soil characterisation

The physical and chemical properties; soil pH, particle size, soil moisture, TOC, TKN and extractable phosphorus of test soils were measured as described in Section 0.

4.2.2 Soil collection and preparation

Uncontaminated (control) soil was collected and prepared as per section 2.2. A concentration gradient of As and Sb was prepared to achieve nominal concentrations of $As_{(Individual)}$ (40, 80, 160, 200, 300 and 400 mg/ kg), $Sb_{(Individual)}$ (40, 80, 160, 300, 600 and 1500 mg /kg) and $As + Sb_{(Combined)}$ (40 + 40, 80 + 80, 160 +160, 200 + 300, 300 + 600 and 400 +1500 mg /kg, respectively). These are referred as treatments.

4.2.3 *Ipomoea aquatica* bioassay

I. aquatica bioassays were established as described in Section 2.3.1. After 35 days, bioassays were terminated and toxicity endpoints (shoot and root length and dry mass) were measured as per Section 2.3.2.

4.2.4 Chlorophyll *a* content of *Ipomoea aquatica* leaves

After 35 days, leaf samples were collected and frozen immediately at -80 °C to determine the chlorophyll content. A known mass (approximately 0.3 g) of leaves was placed in a vial (wrapped in foil) and homogenized using a micropestle in 2 ml of 90% aqueous acetone solution. Samples were transferred to a screw-cap centrifuge tube. The grinder

was rinsed with a minimal amount of 90% aqueous acetone and added (wash solution) to the extraction slurry. The total volume was adjusted to 8 ml with 90% aqueous acetone, centrifuged (20 min at 500 g) and the absorbance measured at 664 and 750 nm by use of a UV-Vis spectrophotometer (UV-1800, Shimadzu). The samples were then acidified with 0.1 M HCl acid and after 90 s the absorbance re-measured at 665 and 750 nm to correct for the presence of pheophytin pigments. The chlorophyll content was calculated using Equation 4.1 (Lorenzen 1967).

$$\text{Chlorophyll } a\ \text{mg/m}^3 = (26.7\ (664_b - 665_a)\ \text{x}\ V_1)/\ (V_2\ \text{x}\ L)) \qquad \text{Equation 4.1}$$

Where: V_1 is volume of extract (L), V_2 is volume of sample (m^3), L is light path length (cm), 664b and 665a are absorbance of the 90% aqueous acetone extract before and after acidification, respectively, and 26.7 is the absorbance correction.

4.2.5 SEP-bioavailable concentration in soil

The sequential extraction procedure (SEP) derived from Wenzel et al. (2001) was performed on soils as described in Section 2.5.1.

4.2.6 Total soil As and Sb in soil and plants

The total As and Sb concentrations in soil and plant samples were measured as per Section 2.5.2 and 2.5.3, respectively.

Analysis was performed using Agilent 7500CE ICP-MS as outlined in Section 2.6. Method detection limits were 0.083 mg As/kg and 0.090 mg Sb/kg. Soils CRM recoveries were within 98-103% (101 ± 2%, n=4) and 66-80% (72 ± 9, n=4) of expected values for As and Sb respectively, and recoveries for plant CRM were within 95-120% (112 ± 19 %, n=4) and 60-85% (75 ± 12 %, n=4) of expected values for As and Sb, respectively.

4.2.7 Data analysis

All data are presented as mean ± SD ($n \leq 3$) (3 pots per concentration and 6 plants per pot) and data analyses were carried out as described in Section 2.7. Significant differences in *I. aquatica* response between treatments were evaluated using one-way ANOVA and Tukey's honest significant difference post-hoc test with an α value of 0.05.

To investigate mixture interactivity, the individual-metalloid EC_{50} values were used to convert soil concentrations into toxic units (TU) by Equation 4.2 (Koppel et al. 2018):

$$\Sigma \mathrm{TU}_i = \sum_{i=1}^{n} \frac{c_i}{EC50_i}$$ Equation 4.2

Where: n is the number of mixture components, c_i is the concentration of an individual component in the mixture, and $EC50_i$ is the concentration of the component i that causes a 50% effect.

The interactive effects of the combined As and Sb treatment on plant growth were determined by calculating the $EC_{50\ (As+Sb)}$ of the combined treatments. A 3-parameter log-logistic model was fitted to the response data with As and Sb concentration expressed as ∑TU (Luan et al. 2008). Mixture interactions were then defined as being concentration additive ($EC_{50\ (As+Sb)}$ = 1 TU), antagonistic ($EC_{50\ (As+Sb)}$ > 1 TU) or synergistic ($EC_{50\ (As+Sb)}$ < 1 TU) (Luan et al. 2008).

4.3 Results and discussion

4.3.1 Soil characterisation and metalloid concentrations

Soil characteristics

Physicochemical characteristics of soil were analysed to ensure homogeneity of test soil. All test soils were a silty sand (30-33% sand, 60-62% silt, 7.5-8.1% clay) and slightly acidic (pH 5.5-6) with average TOC 8.54 ± 0.08%, bioavailable phosphorus 22 ± 7 mg/kg and TKN 565 ± 15 mg/kg.

Total soil As and Sb

Total As and Sb in test soils were measured 14 days after the metalloid spiking, prior to sowing the seeds of *I. aquatica*. Total As in spiked soil ranged from 60 ± 10 to 405 ± 4 mg/kg in $As_{(Individual)}$ and 46 ± 5 to 410 ± 10 mg/kg in $As + Sb_{(Combined)}$ treatments (Table 4.1). Total Sb in soil ranged from 35 ± 5 to 1250 ± 30 mg/kg in $Sb_{(Individual)}$ and 31 ± 2 to 1200 ± 85 mg/kg in $As + Sb_{(Combined)}$ treatments. Similar concentrations have been observed in agricultural soils near Sb mining sites and give a range that allowed for the investigation of interactive effects to toxicity and accumulation (Okkenhaug et al. 2011, Long et al. 2018). In general, As concentrations in the soils surrounding mining areas range from 42-4530 mg/kg and Sb ranges from 74.2-16400 mg/kg (Murciego et al. 2007, Li et al. 2014, Ngo et al. 2016). Therefore, As and Sb concentrations used in this chapter

are environmentally relevant and represent actual As and Sb concentrations found in contaminated agricultural soils.

SEP-bioavailable As and Sb

The SEP-bioavailable As and Sb showed a positive correlation with total soil metalloid concentrations in all treatments (Figure 4.1). The SEP-bioavailable (labile) soil fractions are defined here as the As and Sb associated with non-specific (SO_4^{2-} extractable) and specific (PO_4^{3-} extractable) phases in soils assessed (Wenzel et al. 2001), shown in Figure 4.1 for all treatments. Similar patterns of increasing SEP-bioavailable As with increasing total soil As were observed for both As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments. The percentage of total soil As that was SEP-bioavailable ranged from 23 to 38% in As $_{(Individual)}$ and 20 to 40% in As + Sb $_{(Combined)}$ treatments (Table 4.1 and Figure A3.1). Therefore, the concentrations of SEP-bioavailable As did not change between As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments at the concentrations tested in this research, suggesting that Sb had no impact on the adsorption of As to soil sorption sites at these concentrations.

Similar proportions of SEP-bioavailable Sb (20-22%) were observed at Sb $_{(Individual)}$ treatments ≤500 mg/kg (Table 4.1 and Figure A3.1). However, Sb bioavailability increased to 48% at total soil Sb ≥1200 mg/kg. In As + Sb $_{(Combined)}$ treatments, no significant increase in SEP-bioavailable Sb (19-24%) was observed for total soil Sb ranging from 35-240 mg/kg, but a clear increase of the SEP-bioavailable fraction to 65% was observed when total soil Sb ≥1250 mg/kg. The Chapter 3 of this thesis also reported a significant increase in SEP-bioavailable Sb from 19-30% at concentrations of Sb ≤250 mg/kg to approximately 65% at concentrations of Sb ≥1200 mg/kg in diluted As and Sb co-contaminated mine soils.

Previous studies have shown that As and Sb have different affinities for soil sorption sites (Wilson et al. 2010, Kolbe et al. 2011). Under aerobic conditions, both As and Sb exist in the soil solution as oxyanions, AsO_4^{3-} (tetrahedral) and $Sb(OH)_6^-$ (octahedral). Arsenic has a greater affinity for soil sorption sites than Sb possibly due to AsO_4^{3-} being a smaller molecule in the soil solution compared to SbO_4^{3-}. This is supported by studies showing that As(V) significantly inhibits adsorption of Sb(V) on iron oxides in a dose and ratio dependant-relationship (Wu et al. 2018). These results showed increased SEP-bioavailable Sb at the highest concentration of the As + Sb $_{(Combined)}$ treatment which may be a result of competitive adsorption. However, it should be noted that this conclusion is

based on one high concentration (n=3) and there were no Sb spikes between nominal concentrations, 600 to 1500 mg/kg. Hence, more information would be needed to confirm this trend. These results also showed that the bioavailability of As did not change between individual and combined treatments. This has also been reported by Kolbe et al. (2011), showing that under aerobic conditions there was no effect of $Sb(OH)_6^-$ (Sb(V)) on the sorption of AsO_4^{3-} (As(V)) on to granular iron hydroxide material but sorption of $Sb(OH)_6^-$ decreased by 31% in the presence of 10-fold excess of AsO_4^{3-} at concentrations ranging from 1 to 100 mg/L. Therefore, presence of As in contaminated soil can decrease the sorption of Sb to soil particles and thereby increase Sb bioavailability in soil.

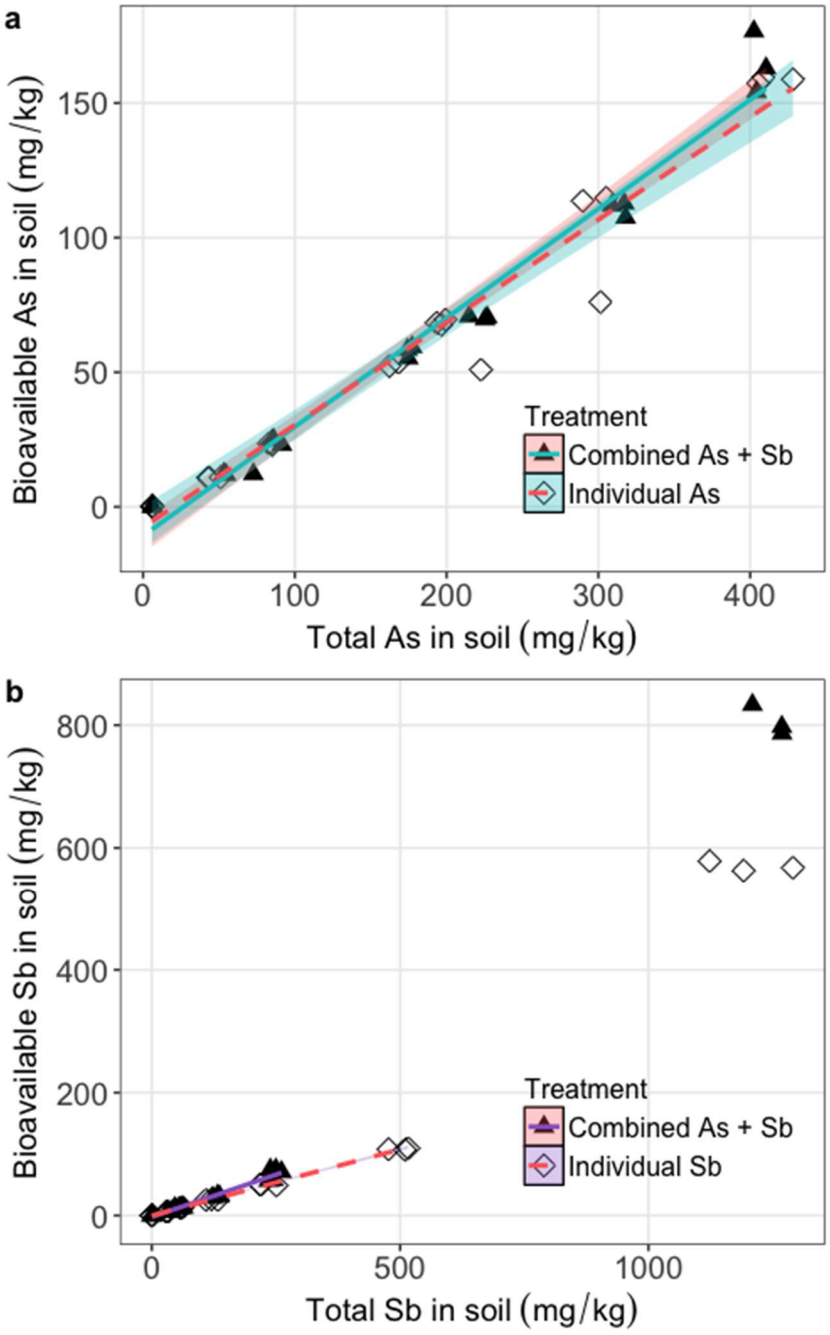


Figure 4.1. The relationship of SEP-bioavailable a). As; and b). Sb concentrations to total soil As and Sb soil concentrations, respectively. The relationship in (b) was linear to concentrations <600 mg/kg, above this there was a departure from linearity and a clear difference between individual and combined treatments (shaded areas represent the 95% confidence intervals).

Table 4.1. Measured total soil, SEP-bioavailable concentrations and percentage bioavailability of As and Sb establishing the bioassays. Individual As and Sb concentrations (labelled I1 – I4) and combined As + Sb (C) concentrations are (labelled C1 – C4) are presented as the mean ± SD, n ≤ 3.

	Total soil concentration		**SEP-Bioavailable**		**% Bioavailability of total soil concentration**	
	As (mg/kg)	**Sb(mg/kg)**	**As (mg/kg)**	**Sb(mg/kg)**	**As%**	**Sb%**
Control	6.1 ± 0.3	0.3 ± 0.03	0.18± 0.005	0.090 ± 0.005	2.9 ± 0.2	46 ± 11
As + Sb (Combined)						
C1	60 ± 10	35 ± 5	12.1 ± 0.2	6.4 ± 0.1	20 ± 3	20 ± 3
C2	90 ± 3	60 ± 10	24 ± 1	13.0 ± 0.3	27.7 ± 0.2	25 ± 5
C3	175 ± 2	130 ± 7	58 ± 2	31 ± 2	33 ± 1	23.9 ± 0.7
C4	220 ± 7	240 ± 5	68.5 ± 0.6	58 ± 1	32 ± 1	24.2 ± 0.7
C5	315 ± 5	250 ± 10	110 ± 3	74 ± 2	35 ± 1	30 ± 2
C6	405 ± 4	1250 ± 30	165 ± 10	800 ± 20	40 ± 3	65 ± 4
As (Individual) and Sb (Individual)						
I1	45 ± 5	30 ± 2	10.83 ± 0.09	6.2 ± 0.2	23 ± 2	20 ± 1
I2	84 ± 1	60 ± 3	23.4 ± 0.2	13.0 ± 0.3	27.7 ± 0.7	21.8 ± 0.8
I3	190 ± 30	120 ± 10	52 ± 1	25.6 ± 0.6	30 ± 5	21 ± 2
I4	195± 2	230 ± 20	69 ± 1	50 ± 1	34.9 ± 0.5	22 ± 2
I5	300 ± 8	500 ± 20	114.2 ± 0.8	108 ± 1	35 ± 8	21.6 ± 0.9
I6	420 ± 10	1200 ± 100	159 ± 1	570 ± 8	38 ± 1	50 ± 4

4.3.2 Toxicity response

I. aquatica is a widely consumed leafy vegetable in many Southeast Asian countries and is easy to grow even in contaminated soil. Heavy metals accumulation in *I. aquatica* has also been observed in some contaminated areas (Göthberg et al. 2002). In this research, no plants died at any concentrations of the As $_{(Individual)}$ or Sb $_{(Individual)}$ treatments. However, in the highest concentration of the As + Sb $_{(Combined)}$ treatment, two out of six plants died in one of the three replicates.

Response of plant growth in different treatments of As and Sb

Shoot moisture content in As $_{(Individual)}$, Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments was consistently between 70-90%, with no trend in response to metal concentrations (Table A3.1). There was no evidence that dehydration contributed to the adverse effects observed in plant physiology during the bioassays.

Shoot and root dry mass significantly decreased to 25% and 55% of control, respectively, at high As concentrations (total: ≥300 mg/kg; SEP-bioavailable: ≥114 mg/kg) (Figure 4.3a and 3g, respectively). However, shoot and root dry mass showed no significant decrease compared to controls following exposure to Sb $_{(Individual)}$ treatment (Figure 4.3b and 3h, respectively). Exposure to the As + Sb $_{(Combined)}$ treatment, significantly decreased shoot and root dry mass to as low as 14% and 46% of control, respectively at the highest concentrations (Figure 4.3c and 3i, respectively).

Shoot length significantly decreased following exposure in the As $_{(Individual)}$ treatment to a minimum of 22% compared to control at high As concentrations (total: ≥300 mg/kg; SEP-bioavailable: ≥114 mg/kg) (

Figure **4.2** and Figure 4.3d). However, root length only decreased at the highest As concentration (Figure 4.3j). The Sb $_{(Individual)}$ treatment had no impact on either shoot or root length (Figure 4.3e and 3k, respectively). In the As + Sb $_{(Combined)}$ treatment, shoot length significantly decreased to 20% of control at all exposure concentrations, whereas a significant decrease in root length was observed only at the highest concentration (SEP-bioavailable: 165 mg/kg and 800 mg/kg, respectively) (Figure 4.3f and 3l, respectively).

Previous studies testing As toxicity observed a similar decrease in shoot and root dry mass and length at lower As soil concentrations compared this research. For example, to

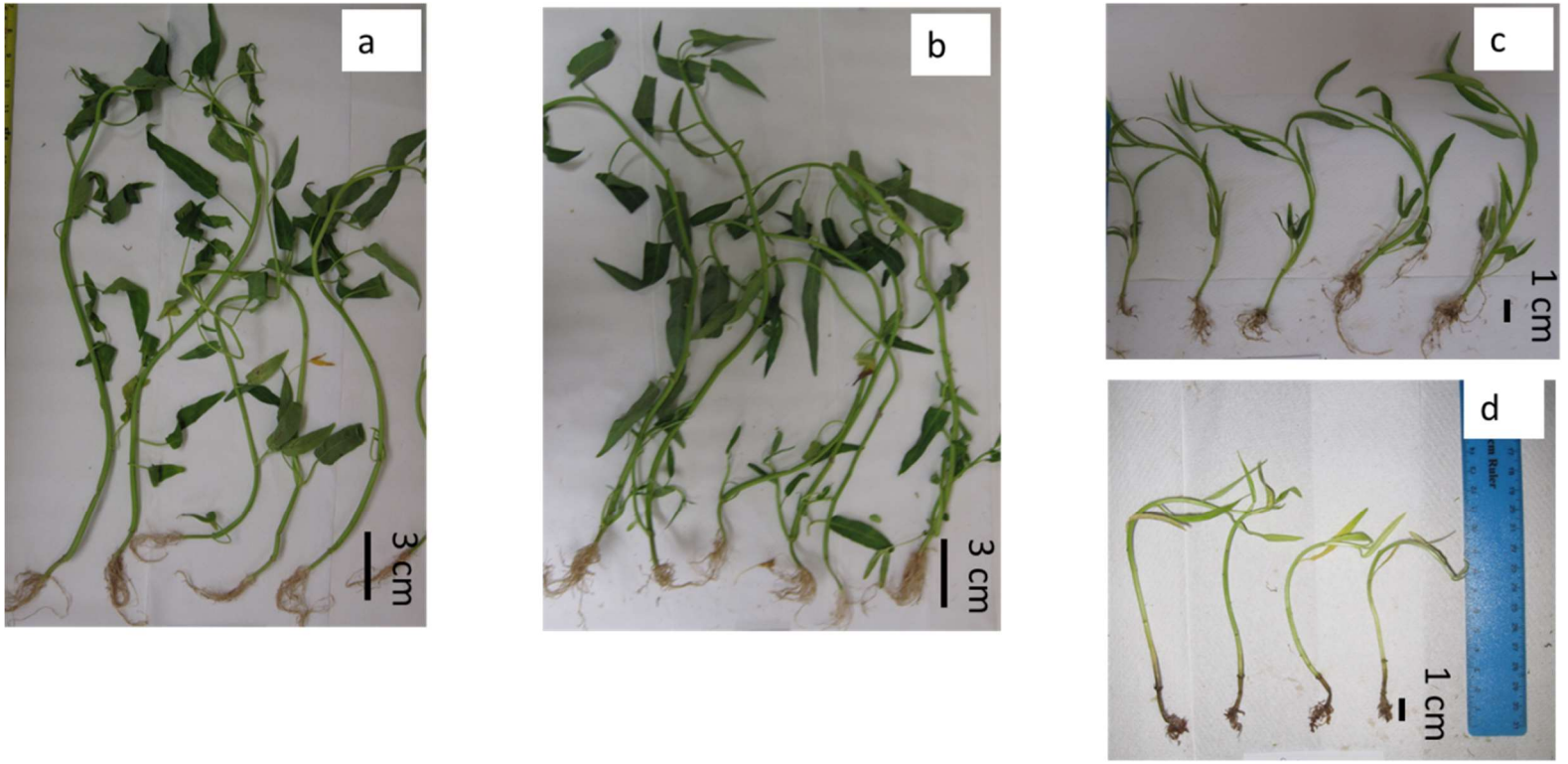


Figure 4.2. Morphology of *Ipomoea aquatica* in the contaminated soil with high As and Sb concentrations: a). Control; b). 1200 mg Sb/kg; c). 415 mg As/kg; d). 405 mg As/kg + 1250 mg Sb/kg.

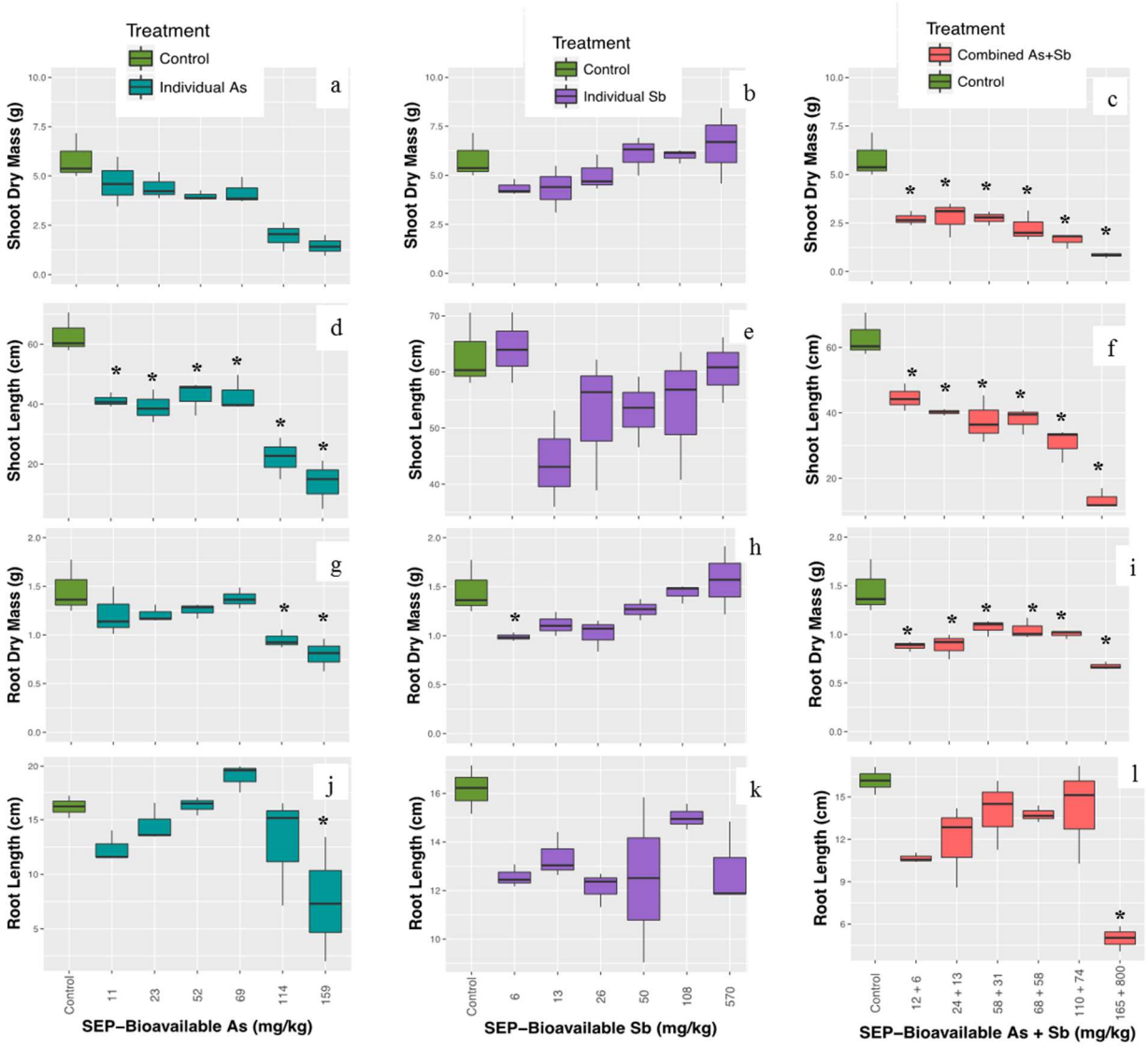


Figure 4.3. Individual and combined toxicity of As and Sb on growth of *Ipomoea aquatica*. * indicate significant difference between control and treatment.

wheat seedlings at concentrations as low as 20 mg/kg or in anaerobic conditions to rice (*Oryza sativa*) at high As concentrations (Shri et al. 2009, Nath et al. 2014). Arsenic has also been shown to stimulate the growth of plants such as maize, peas, potatoes, rye, tomato, soybean and cotton. Arsenic may stimulate the plant growth as As(V) can disturb the phosphate-dependent metabolic processes by forming unstable adenosine di-phosphate arsenate (ADP-As) complexes which disrupts the energy production in plant cells leading to cell death (Meharg et al. 2002, Garg et al. 2011, Finnegan et al. 2012). Further, As(V) can accumulate in the cytoplasm and reduce to As(III) by arsenate

reductase and be bound by sulphydryl (-SH) groups of peptides in enzymes and tissue proteins, causing them to lose their functionality, eventually leading to cell death (Meharg et al. 2002, Zhao et al. 2009, Garg et al. 2011, Abbas et al. 2018). Moreover, As accumulation in plants leads to the generation of reactive oxygen species (ROS) such as $O2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2, which are known strong oxidising agents and cause lipid peroxidation (Shri et al. 2009, Garg et al. 2011, Abbas et al. 2018). These effects are generally observed as phytotoxic symptoms including leaf wilting, cell plasmolysis and root discolouration, inhibited root extension, cell growth, reduced fertility, reduced yield and reduced fruit production (Naidu et al. 2006, Finnegan et al. 2012).

Antimony, on the other hand, had little to no effect on shoot and root dry mass and length in this research, with only one concentration showing a significant decrease in root dry mass compared to the control. Plants can uptake Sb species from the soil in the form of Sb(III) and Sb(V) and induce toxic effects. The toxic effects of Sb(III) mainly includes inhibition of photosynthetic electron transfer, carbonic anhydrase activity of photosystem II and glutathione reductase activity of chloroplasts (Karacan et al. 2016, Ortega et al. 2017). Some studies also showed that toxicity of Sb may be associated with increased ROS production and uptake of essential nutrients (Ca^{2+} and Na^{+}) from the soil (Cooper et al. 2009, Feng et al. 2013). Despite these mechanisms of toxicity, Feng et al. (2013) found no decrease in shoot dry mass in rice plant leaves at Sb concentration up to 66 mg/kg. In contrast, Pan et al. (2011) showed that shoot length/ dry mass of maize seedlings were significant inhibited when soil Sb equal to or exceeded 50 mg/kg. The same study showed that root length and the number of roots decreased with increasing soil Sb concentrations. Thus, it appears that the toxicity of Sb is plant specific.

Overall, As and Sb co-contaminated soil had a greater impact on *I. aquatica* shoot dry mass and shoot length compared to single-As exposure especially at the highest As and Sb concentration. Chapter 3 showed that soil historically contaminated with As and Sb also caused toxicity to *I. aquatica* with a significant decrease in shoot dry mass, root dry mass, shoot length and root length with increasing As and Sb concentrations.

4.3.3 Mixture toxicity and interactivity

The response of plant growth at different metalloid treatments showed that only shoot length and dry mass of *I. aquatica* in $As_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments

exhibited a significant dose-response relationship relative to total soil and SEP-bioavailable concentrations (Table 4.2). Although there were no obvious differences observed when fitting the log-logistic model between total and SEP-bioavailable concentration, much lower EC values were obtained for SEP-bioavailable concentration in comparison with total concentration. This suggests that both total and SEP-bioavailable concentrations of a contaminant are suitable to predict toxicity to *I. aquatica* in these soils.

Table 4.2. Effect concentration estimates that cause a 10% and 50% (**EC_{10}** and **EC_{50}**, with 95% confidence intervals) decrease in shoot dry mass and length of *Ipomoea aquatica* exposed to As and Sb individually and combined relative to total soil metalloid concentration and SEP-bioavailable concentrations. Sb $_{(Individual)}$ EC_{10} and EC_{50} values are not available due to no observed toxicity in those treatments.

	As (Individual)		**As + Sb (Combined)**	
	EC_{10} (mg/kg)	**EC_{50} (mg/kg)**	**EC_{10} (mg/kg)**	**EC_{50} (mg/kg)**
Total soil concentration				
Shoot dry mass	50 (6-96)	240 (186-296)	5 (0-13)	90 (55-126)
Shoot length	15 (0-33)	200 (140-270)	20 (0-39)	220 (152-285)
SEP-bioavailable concentration				
Shoot dry mass	10 (0-22)	81 (56-106)	0.4 (0-1.2)	20 (7.4-33)
Shoot length	2 (0-6)	65 (38-94)	3 (0-7)	72 (43-101)

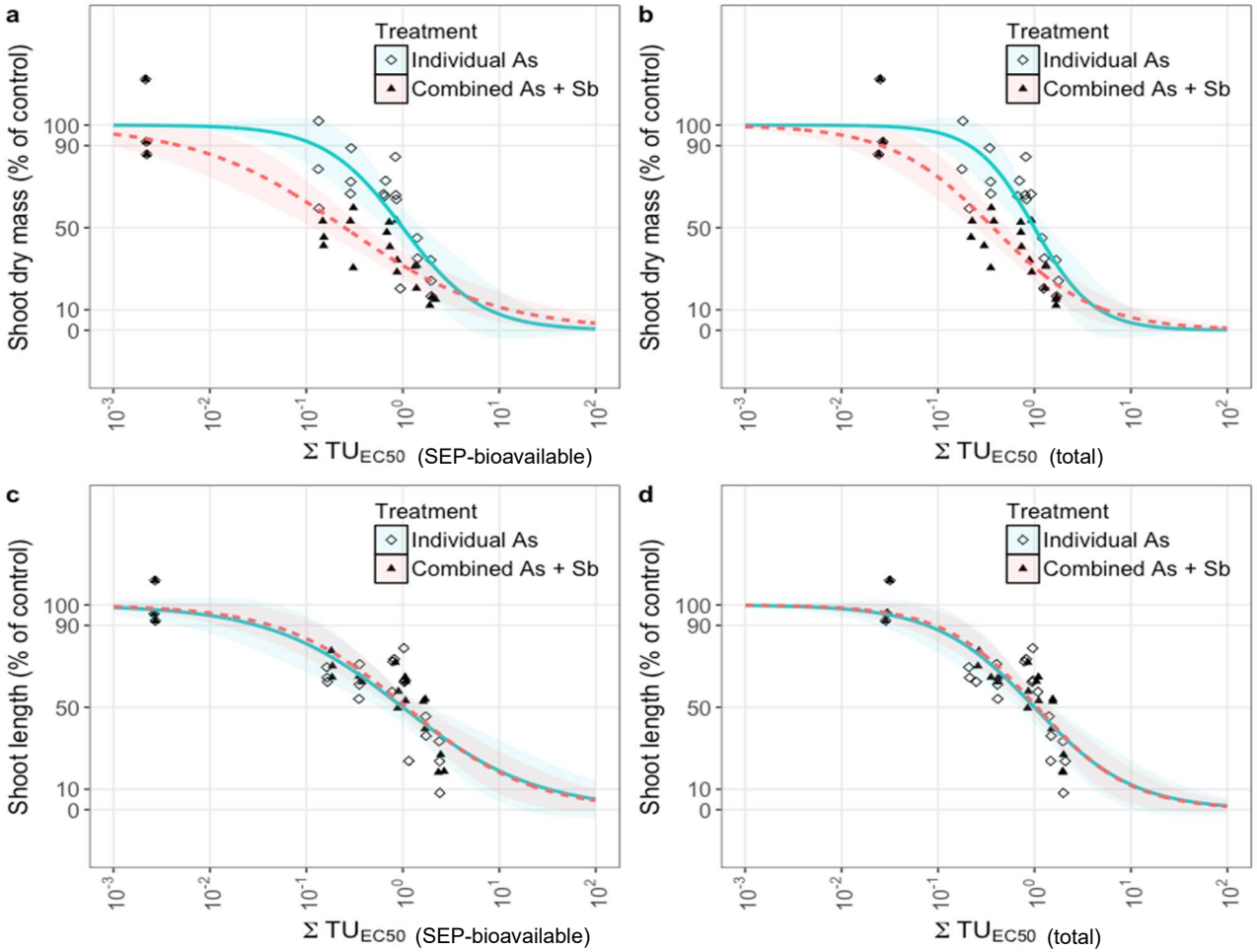


Figure 4.4. Dose-response curves of individual As and combined As + Sb on *Ipomoea aquatica* shoot dry mass (a, b) and length (c, d) after the 35-day bioassay, relative to SEP-bioavailable (a, c) and total metalloid As (b, d) concentrations in soil, respectively. Concentrations are expressed as toxic units, see Figure 4.3 for boxplots of toxicity endpoints by As and Sb concentrations.

This research used metalloid-spiked soils with a 14 d equilibration period, which is likely why both total and SEP-bioavailable concentrations were suitable to predict toxicity. Chapter 3 of this thesis showed that SEP-bioavailable concentration is better in predicting the toxicity of As in highly weathered contaminated soils, compared to metalloid-spiked soil, because bioavailable concentrations better represent the fraction of metalloid responsible for plant uptake. This may be due to the strengthened binding of metalloids to various soil fractions in historically aged soils compared to recently contaminated (Section 3.3.2).

The lower EC_{10} and EC_{50} obtained for shoot dry mass showed that the As + Sb $_{(Combined)}$ treatment was more toxic than the As $_{(Individual)}$ treatment at equivalent concentrations of either total soil or SEP-bioavailable As concentration (Table 4.2), despite no toxicity observed from the Sb $_{(Individual)}$ treatment. Although the EC_{10} and EC_{50} for shoot length in the As $_{(Individual)}$ treatment was lower than As + Sb $_{(Combined)}$ treatment, the 95% CI showed that these EC values are not significantly different between the two treatments (Table 4.2). On the other hand, the concentration-response curves of As $_{(Individual)}$ treatment intersected the lines of EC_{50} at 1 TU. When considering the interactive effect of As and Sb on shoot dry mass, there was a slight shift to the left in the As + Sb $_{(Combined)}$ concentration-response curve compared to As $_{(Individual)}$ treatment (EC_{50} of As + Sb $_{(Combined)}$ was less than 1 TU) (Figure 4.4a and b). This suggests that the mixture of As + Sb had a synergistic toxicity on the shoot dry mass of *I. aquatica* relative to both total soil and SEP-bioavailable As concentrations. However, for shoot length (Figure 4.4c and d), there was no shift in the As + Sb $_{(Combined)}$ concentration- response curve compared to As $_{(Individual)}$ treatment (EC_{50} of As + Sb $_{(Combined)}$ was equal to 1 TU). This suggests that the mixture of As + Sb had an additive interactive effect on the shoot length of *I. aquatica.*

This research showed that exposure of *I. aquatica* to As and Sb in co-contaminated soils had a greater effect on the shoot dry mass than shoot length. This may be caused by Sb placing a greater burden on plant detoxification mechanisms. A major mechanism of As and Sb detoxification in plants is binding to PCs, sequestration to vacuoles and methylation (Wang et al. 2015). Further, As and Sb induce reactive oxygen species which are detoxified by PCs or other antioxidant molecules (Feng et al. 2011, Feng et al. 2013). Thus, the synergistic toxicity observed may have resulted from competition for binding to PCs and increased induction of ROS within the cells, however, further targeted studies are needed to explore these interactions.

4.3.4 Effects on photosynthetic efficiency and chlorophyll a concentration

Exposure to As and Sb individually or combined had no effect on photosynthetic efficiency (Figure A3.2), with similar maximum photosynthetic efficiency (Fv/Fm) for the control (0.83) and treatments (0.81 to 0.84 for all treatments). Chlorophyll *a* was negatively correlated with SEP-bioavailable As and Sb concentrations in the combined treatments (Figure A3.3) whereas it was positively correlated to As in the As $_{(Individual)}$ treatment. In support of this, Päivöke et al. (2001) showed that increasing As concentrations in soil increased the chlorophyll *a* content in *Pisum sativum L.* (Päivöke et al. 2001). On the other hand, chlorophyll *a* showed no correlation with Sb in the Sb $_{(Individual)}$ treatment. However, contrasting observations have been reported by Zhou et al. (2018) who showed that increasing Sb concentrations in soils decreased the chlorophyll *a* content in *Acorus calamus*. It is unclear what caused the decrease to chlorophyll *a* content in the combined treatment, but it does not appear to be related to changes in the bioavailable fraction of As or Sb (Figure 4.1). It may be a result of increased Sb accumulation in shoots (Figure 4.5); however, more information is needed to confirm observed differences in Sb accumulation between individual and co-contaminated treatments.

4.3.5 Accumulation of As and Sb in edible parts

Similar trends of As accumulation in edible parts (shoots) were observed when exposed to As $_{(Individual)}$ (R^2= 0.991) and As + Sb $_{(Combined)}$ (R^2=0.993) treatments. In As $_{(Individual)}$ treatments, As concentrations in shoots linearly increased from 14 to 80 mg/kg when exposed to SEP-bioavailable As concentrations ranging from 12 - 69 mg/kg (Figure 4.5a) followed by a decrease in shoot As at SEP-bioavailable As concentration greater than 110 mg/kg. A similar pattern was observed in *I. aquatica* exposed to As + Sb $_{(Combined)}$ treatment with As in shoots increasing from 16 to 80 mg/kg as SEP-bioavailable As increased up to 69 mg/kg, followed by a decrease in shoot As at SEP-bioavailable As concentrations greater than 68 mg/kg. This decrease in both As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments may be due to the decreased plant physical growth (dry mass), as shown in

Figure 4.2. Arsenic accumulation in the shoots was not affected by the presence of Sb, even at high concentration (>1200 mg Sb/kg).

In the Sb $_{(Individual)}$ treatment, accumulation in the shoots slightly increased from 0.8 to 14 mg/kg at SEP-bioavailable Sb concentrations ranging from 6 to 570 mg/kg (Figure 4.5b). A similar trend was observed for Sb in As + Sb $_{(Combined)}$ treatment up to a SEP-bioavailable Sb concentration of 74 mg/kg. In the As + Sb $_{(Combined)}$ treatment at the highest concentration (SEP-bioavailable Sb of 800 mg/kg) there was a large increase in shoot Sb accumulation, to 100 mg/kg (Figure 4.5b). This may reflect the preferential binding of As over Sb to soil binding sites leading to an increase of Sb uptake in the presence of As (see Figure 4.5b). However, care must be taken when considering these results as the highest SEP-bioavailable Sb concentration was higher in the As + Sb $_{(Combined)}$ treatment compared to the Sb $_{(Individual)}$ treatment. Furthermore, Sb accumulation at SEP-bioavailable concentrations up to approximately 70 mg Sb/kg were equivalent, with only the higher concentrations showing a difference. In general, *I. aquatica* shoots accumulated more As than Sb in the As + Sb $_{(Combined)}$ treatment. This could be due to higher bioavailability of As in the co-contaminated soils compared to Sb (shown in Table 4.1).

This study showed that As accumulation in *I. aquatica* was not affected by As and Sb co-contamination, but Sb accumulation increased only at high concentrations. Some studies have investigated the influence of As and Sb co-contamination on the accumulation of As and Sb in plants. Fu et al. (2016) observed different accumulation of As (2.32 mg/kg) and Sb (14.1 mg/kg) in vegetables grown in co-contaminated mining soils ranging between 62.9 mg As/kg and 1337 mg Sb/kg. In support of these results, Feng et al. (2011) showed that the presence of As and Sb increased Sb uptake by *P. cretica,* especially at low As concentrations. Similar results also have been reported for *P. vittata*, with increased Sb uptake in the presence of As (Muller et al. 2013).

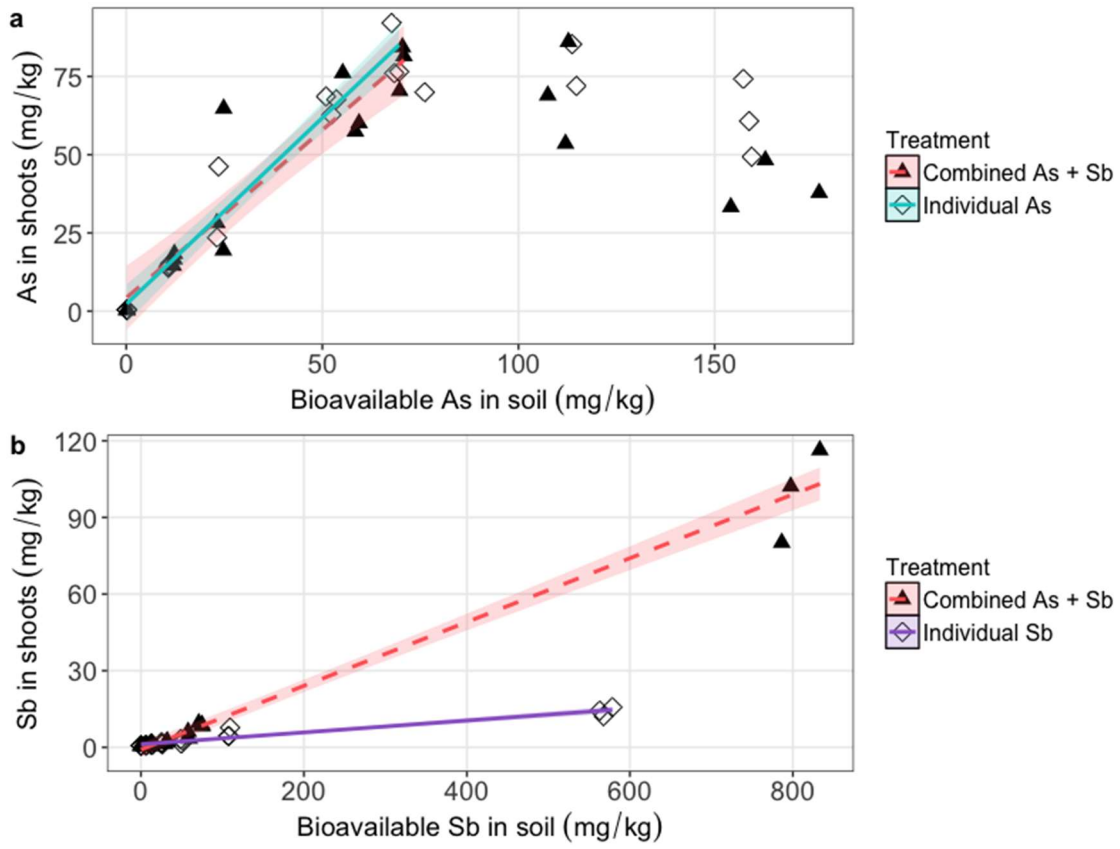


Figure 4.5. The accumulation of a). As and b). Sb in *Ipomoea aquatica* shoots at the end of the bioassay compared to SEP-bioavailable concentrations in soils from individual As and Sb and combined As + Sb treatments. The linear regression in a) was only applied to the linear portion of the response (shaded areas represent the 95% confidence intervals).

4.4 Conclusion

This chapter investigated the interactive effect of As and Sb on their bioavailability in soils and toxicity and accumulation in *I. aquatica.* The combined treatment had no effect on As bioavailability or accumulation. Similarly, there was no difference in SEP-bioavailable Sb or Sb accumulation in shoots compared to the individual treatment, except at the highest concentration which showed greater Sb bioavailability and accumulation.

The $As_{(Individual)}$ treatment resulted in plant toxicity but the $Sb_{(Individual)}$ treatment did not. In the combined treatment, there was synergistic toxicity (EC_{50} less than 1 TU) to shoot dry mass and additive toxicity (EC_{50} equal to 1 TU) to shoot length. The synergistic toxicity was not explicitly explained by the accumulation of As or Sb, which may be a result of changes to internal cellular processes such as detoxification mechanisms. However, further studies are needed to identify possible mechanisms of toxicity and interactions during co-exposure, especially at high Sb concentrations where changes to SEP-bioavailable Sb and accumulation were evident.

Arsenic and Sb often co-occur in agricultural fields near mining sites, posing a greater risk than what would be expected from exposure to the contaminants individually. However, agricultural fields may also expose to other competitive ligands, such as PO_4^{3-} through fertilisers, possibly leading to competitive interactions on bioavailability, accumulation and toxicity.

Chapter 5. Influence of soil PO_4^{3-} on accumulation and toxicity of As and Sb in *Brassica chinensis* grown in individually and co-contaminated soils.

Egodawatta, L.P., Holland, A., Koppel, D., Jolley, D.F. 2020. Influence of soil phosphate on accumulation and toxicity of As and Sb in choy sum cultivated in individually and co-contaminated soils. *Environmental Toxicology and Chemistry*. DOI: 10.1002/etc.4708

"**LPE**: Conceived the experiment design and carried out the experiment, formal data analysis, prepared all tables and figures, wrote the original manuscript draft. **DK**: Supervision, contributed to the statistical data analysis and preparing figures using R studio software, preparing editing the manuscript. **AH:** Supervision, contributed to the statistical data analysis and editing the manuscript. **DFJ**: Supervision, provided resources and funds, contributed to review & editing of the manuscript.

5.1 Introduction

The rapid expansion of the global population has driven an increase in food production. This has led to a significant increase in the use of phosphate-based fertilisers. Phosphate is a macronutrient for plant growth, being an essential component of biomolecules including nucleic acids, phospholipids and adenosine triphosphate (Schachtman et al. 1998).

In Chapter 4, co-contamination of As and Sb was shown have synergistic phytotoxicity and increased accumulation in agricultural plants. The main factors controlling As and Sb mobility and speciation are soil redox potential, pH and the presence of competitive ions such as PO_4^{3-}. The oxyanions of P(V) and As(V) have tetrahedral structures and display very similar physical and chemical behaviours under aerobic environments (Strawn 2018). As a result, PO_4^{3-} and AsO_4^{3-} compete for the same soil sorption sites and can result in an increased As bioavailability (Anawar et al. 2018). Some studies have shown that the presence of PO_4^{3-} in soil can partially decrease $Sb(OH)_6^-$ adsorption on soil sorption sites and thereby increase Sb bioavailability in soil (Biver et al. 2011, Xi et al. 2011).

Phosphate and AsO_4^{3-} share a common transport pathway (inorganic phosphate transporters (PHT) in plant roots. Competition between PO_4^{3-} and AsO_4^{3-} at the plant root surface has been observed in a range of studies (Quaghebeur et al. 2004, Pigna et al. 2009, Zhang et al. 2017, Anawar et al. 2018). Some studies reported that these transporters had a greater affinity for PO_4^{3-} than AsO_4^{3-} (Pigna et al. 2009, Anawar et al. 2018). Xenidis et al. (2010) showed that As uptake by dwarf beans increased with increasing soil PO_4^{3-}. In contrast, Klaber et al. (2014) showed that addition of PO_4^{3-} fertilisers did not enhance As accumulation in rice cutgrass (*Leersia oryzoides* Sw.) and tall fescue (*Festuca arundinacea* Schreb.), suggesting that the uptake kinetics of PO_4^{3-} and AsO_4^{3-} in plants may be dependent on the species. Unlike As(V), there is no evidence that Sb(V) uptake from soils to plants occurs via the phosphate transport system. Instead, Tschan et al. (2009) suggests that Sb(V) uptake may be primarily associated with the apoplastic pathway, entering cells via anion transporters.

Arsenic toxicity causes tissue damage and cell death which in turn lowers growth rates and can increase mortality in plants (Gunes et al. 2009). The presence of PO_4^{3-} in soil may enhance the uptake of As in plant tissues while ameliorating adverse effects of As

on plants, such as decreased growth. For example, the addition of PO_4^{3-} was shown to increase the uptake of As in chickpea (*Cicer arietinum*) and Chinese brake (*Pteris vittata*) plants but alleviate As phytotoxicity by partially protecting the membranes from As-induced oxidative stress (Tu et al. 2003, Gunes et al. 2009). This may give the appearance of a healthy plant which may still have accumulated contaminants. No information is available on toxic effects of Sb in the presence of PO_4^{3-} in soil. Therefore, it is important to study how PO_4^{3-} interacts with oxyanions of Sb(V) in soils and how this may affect the bioavailability, accumulation and toxicity of Sb to plants, particularly in environmentally realistic scenarios of As and Sb co-contamination.

This chapter investigates the influence of two soil PO_4^{3-} concentrations (low and high) on the bioavailability, bioaccumulation and toxicity of As and Sb in individually (As $_{(Individual)}$, Sb $_{(Individual)}$) and combined (As + Sb $_{(Combined)}$) contaminated soils. This was investigated using choy sum (*Brassica chinensis* var. *parachinensis)*, a commonly consumed leafy vegetable, particularly in Chinese cuisine. It also has been reported that leafy vegetables tend to accumulate more As and Sb than other vegetables and, as such, choy sum was chosen as it is easy to grow in any fertile soil, is commercially important, and easy to maintain (Fu et al. 2016). Please note that writing PO_4^{3-} and AsO_4^{3-} in this chapter has no implications concerning the actual speciation of the elements.

5.2 Methods

5.2.1 Soil preparation

The control soil was spiked as per Section 2.2 to obtain three different soil treatments; i) As $_{(Individual)}$ ii) Sb $_{(Individual)}$ and iii) As + Sb $_{(Combined)}$. Each treatment had four nominal concentrations of As $_{(Individual)}$ (80, 200, 300 and 400 mg/ kg), Sb $_{(Individual)}$ (80, 300, 600 and 1500 mg /kg) and As + Sb $_{(Combined)}$ (80 + 80, 200 + 300, 300 + 600 and 400 +1500 mg /kg, respectively) and each concentration had three replicates.

Two bioassays were established using the same base soils (to minimise experimental variability), with low PO_4^{3-} soil having ~100 mg P/kg and high PO_4^{3-} soil having ~500 mg P/kg. Each of the bioassay soils was amended with either low or high PO_4^{3-} $(NH_4H_2PO_4)_{(aq)}$ concentrations and allowed to equilibrate for 14 days, replicating a recently contaminated soil scenario. Soil subsamples from each replicate were collected and characterised.

5.2.2 Soil characterisation

The physical and chemical properties; soil pH, particle size, soil moisture, TOC, TKN and extractable phosphorus were measured as described in Section 0.

5.2.3 *Brassica chinensis* bioassay

A 40-day bioassay was established using choy sum (*B. chinensis*) where plant root and shoot length and biomass were measured to evaluate toxicity. Seeds were sourced and germinated as per Section 2.3. Twelve seeds were sown in three replicate soil pots per concentration per treatment (day 0) and placed inside a growth chamber (Thermoline Scientific Incubator) and conducted for 40 days as discussed in Section 2.3. Forty days was chosen for termination as *B. chinensis* usually takes approximately 4-6 weeks to mature.

After 40-days (at plant maturity) plants were harvested, rinsed with high purity water, sectioned into roots and shoots and toxicity endpoints (shoot and root length and dry mass) were measured as discussed in Section 2.3.2.

5.2.4 Soil and plant analysis

The sequential extraction procedure (SEP) was derived from Wenzel et al. (2001) as outlined in Section 2.5.1. Total As and Sb concentrations in test soil samples were determined before starting the bioassays (low and high PO_4^{3-}soil) and the methods are discussed in Sections 2.5.2. Total As and Sb concentrations in plant samples were determined as discussed in Sections 2.5.3.

All soil and plant extracts were analysed for As and Sb by Thermo Scientific iCAP-Q ICP-MS as detailed in Section 2.6. Method detection limits for plant digests were 0.064 mg As/kg and 0.077 mg Sb/kg and soil digests were 2.622 mg As/kg and 0.29 mg Sb/kg. Soils CRM recoveries were within 76-105% (mean = 83 ± 12%, n=4) and 83-90% (mean = 85 ± 4%, n=4) of expected values for As and Sb in soils, respectively, and within 96-130% (mean = 121 ± 20%, n=8) and 60-84% (mean = 85 ± 24%, n=8) of expected values for As and Sb in plants, respectively.

5.2.5 Data Analysis

All data are presented as mean ± SD $n \leq 3$ (3 pots per treatment, 6 plants per pot) and were performed using R where significance was defined at an $\alpha = 0.05$ and details as per

Section 2.7.

Bioaccumulation factors (BAF) were used to evaluate the transfer of metalloids from soil to plants. These were calculated using Equation 5.1:

$$BAF = \frac{As\ or\ Sb\ in\ roots}{SEP - bioavailable\ As\ or\ Sb\ concentrations} \quad \text{Equation 5.1}$$

Translocation of metalloids from roots to shoots (expressed as translocation factor, TF) was also calculated using Equation 5.2:

$$TF = \frac{As\ or\ Sb\ in\ Shoots}{As\ or\ Sb\ in\ Roots} \quad \text{Equation 5.2}$$

5.3 Results and discussion

5.3.1 Soil Characterisation

All test soils were a silty sand and moderately acidic with a pH of 5.4. The TOC and TKN contents were 8.5% and 500 mg/kg, respectively, for both low and high PO_4^{3-} experiments. The sodium bicarbonate extractable P concentration varied from 43-88 mg/kg in low PO_4^{3-} soil and 100-180 mg/kg in high PO_4^{3-} soil. All the soil characteristics are summarized in Table A4.1.

The As and Sb concentrations used in this research are representative of concentrations measured in real contaminated concentrations in agricultural fields and thereby, to assess the potential risks of using PO_4^{3-} containing fertilisers. The total As in test soils covered a wide range of concentrations, between 55-335 mg/kg and 53-330 mg/kg in $As_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments, respectively (Table 5.1). These concentrations are within the range of As concentrations in agricultural fields near mining areas which may range between 138-379 mg/kg (Okkenhaug et al. 2011, Abad-Valle et al. 2018, Long et al. 2018). Total Sb concentrations ranged between 80-2220 mg/kg and 163-1680 mg/kg for the $Sb_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments, respectively. In agricultural fields in the vicinity of Sb mining sites, Sb concentrations ranged between 19-8733 mg/kg (Cidu et al. 2014, Fu et al. 2016).

5.3.2 Sequentially extractable As and Sb in soils

Generally, the SEP-bioavailable As fraction increased with increasing soil concentrations (Figure 5.1a and b, respectively). In both $As_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments,

higher amount of SEP-extractable As were observed in high PO_4^{3-} soils compared to low PO_4^{3-} soils (Figure A4.1). In As $_{(Individual)}$ treatment, SEP- bioavailable As (defined as the sum of non-specifically sorbed and specifically sorbed) showed no difference between low and high PO_4^{3-} soils (Figure 5.1a). In As + Sb $_{(Combined)}$ treatment, SEP-bioavailable As was considerably lower in the two highest concentrations of high PO_4^{3-} soil compared to low PO_4^{3-} soil (Figure 5.1b). However, these results did not show a clear competition between AsO_4^{3-} and PO_4^{3-} for sorption sites in soil. Previous studies showed that under aerobic conditions, As and P exist in +5 oxidation state in the form of oxyanions (AsO_4^{3-} and PO_4^{3-}), which have similar atomic radius of 2.48 Å for AsO_4^{3-} and 2.38 Å for PO_4^{3-}, and show similar chemical behaviour (Tighe et al. 2007, Tawfik et al. 2011). Due to the relatively small differences between the two structures of AsO_4^{3-} and PO_4^{3-}, they should compete for sorption sites on soil particles and substitute each other in biogeochemical reactions including adsorption/desorption and precipitation/dissolution reactions (Rivas-Pérez et al. 2015). Further, Rivas-Pérez et al. (2015) reported that PO_4^{3-} has a greater binding affinity for Fe and Al (hydr)oxides than AsO_4^{3-}, and should outcompete As for these soil binding phases, subsequently increasing the bioavailability of As.

Similar to As, SEP-extractable Sb increased with increasing soil Sb concentrations (Figure 5.1c and d). For Sb $_{(Individual)}$ treatment, no increase in SEP-extractable Sb was observed at high PO_4^{3-} soil compared to low PO_4^{3-} soil for most of the concentrations except at 2220 mg/kg. For As + Sb $_{(Combined)}$ treatment, no increase in SEP-extractable Sb was observed at any concentration during high PO_4^{3-} addition. Although, Sb was primarily associated with amorphous Fe and Al oxides, a slight decrease in this fraction (with an increase in the crystalline Fe and Al oxides) was observed at high PO_4^{3-} soil in both treatments (Figure A4.1). Further, for both Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments, SEP-bioavailable Sb appeared to be similar between low and high PO_4^{3-} soil, except for SEP-bioavailable Sb in As + Sb $_{(Combined)}$ treatment C4 (≥1680 mg/kg Sb) (Figure 5.1). In contrast, Feng et al. (2013) reported that addition of PO_4^{3-} amendments was observed to effectively mobilise Sb in Sb-contaminated soils. While it has been reported that the presence of both PO_4^{3-} and AsO_4^{3-} may decrease Sb adsorption to soil sorption sites by competitive binding, increasing $Sb(OH)_6^-$ bioavailability (Kolbe et al. 2011, Arco-Lázaro et al. 2016, Rouwane et al. 2016, Qi et al. 2017), the results of this chapter did not support this.

Table 5.1. Soil As and Sb concentrations in the high and low PO_4^{3-} bioassays with *Brassica chinensis* var. *parachinensis*. Total soil concentrations were measured at the start of the bioassays, and SEP-bioavailable As and Sb were measured at the end of bioassay. Individual (I) As and Sb concentrations (labelled I1 – I4) and combined (C) (As + Sb concentrations (labelled C1 – C4) are presented as the mean ± SD, $n \leq 3$.

Treatment	Total soil concentration		SEP-bioavailable concentration at the end of each bioassay			
	As (mg/kg)	Sb (mg/kg)	As (mg/kg)		Sb (mg/kg)	
			Low PO_4^{3-}	High PO_4^{3-}	Low PO_4^{3-}	High PO_4^{3-}
Control	3.9 ± 0.2	0.47 ± 0.01	0.10 ± 0.01	0.05 ± 0.01	< 0.077	0.06 ± 0.01
As + Sb (Combined) treatment						
C1	53 ± 2	163 ± 0.1	12 ± 2	7.5 ± 0.9	3.3 ± 0.5	3.4 ± 0.3
C2	160 ± 15	249 ± 0.03	33 ± 2	30 ± 12	17 ± 1	17 ± 4
C3	240 ± 7	244 ± 0.06	60 ± 3	36 ± 3	20 ± 4	19 ± 2
C4	330 ± 20	1680 ± 0.1	87 ± 4	58 ± 1	136 ± 8	100 ± 5
As (Individual) or Sb (Individual) treatment						
I1	55 ± 3	80 ± 5	12 ± 1	15 ± 8	2.6 ± 0.1	5.1 ± 0.2
I2	170 ± 4	360 ± 15	32 ± 0.1	27 ± 6	16 ± 1	16 ± 1
I3	240 ± 7	830 ± 30	53 ± 4*	38 ± 2*	34 ± 1	32 ± 2
I4	335 ± 4	2220 ± 100	73 ± 5	71 ± 1	68 ± 1	72 ± 2

* Only two replicates were used for the mean calculation due to a measurement error.

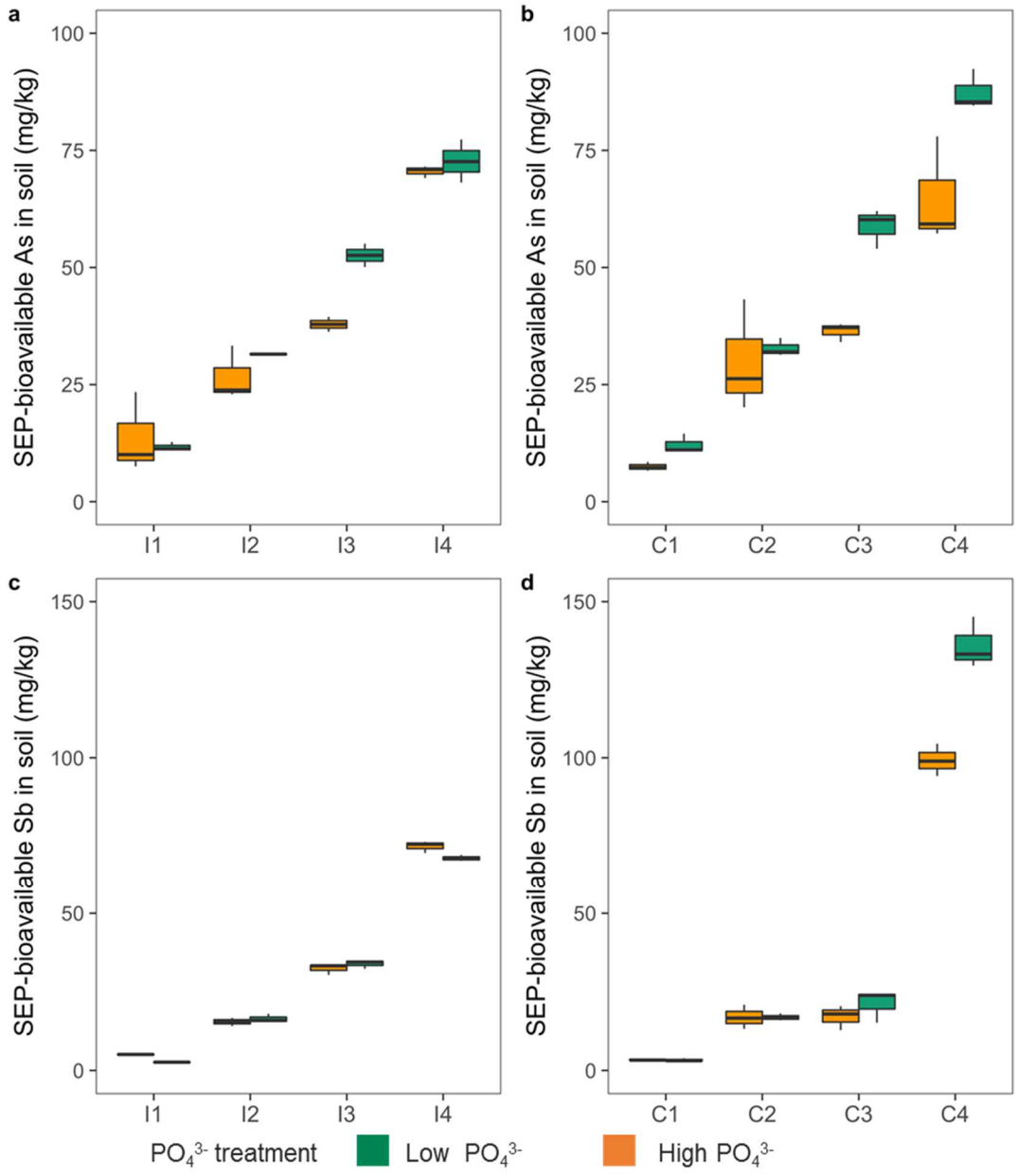


Figure 5.1. The distribution of SEP-bioavailable a). As in As $_{(Individual)}$ b). As in As + Sb $_{(Combined)}$ c). Sb in Sb $_{(Individual)}$ d). Sb in As + Sb $_{(Combined)}$ at low and high PO_4^{3-} levels, mean ± SD, n ≤ 3**.** Individual As and Sb concentrations (labelled I1 – I4) and combined As + Sb concentrations are (labelled C1 – C4) given in Table 5.1.

5.3.3 Influence of soil PO_4^{3-} on the toxicity induced by As and Sb

The response of *B. chinensis* shoot and root dry mass in As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments at different PO_4^{3-} concentrations are shown in

Figure 5.2. For both treatments, shoot and root dry mass in low PO_4^{3-}soil significantly decreased to 30% and 20% of controls, respectively, at the highest As concentrations (I4 and C4, as per Table 5.1). However, for both As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments, As showed no toxicity to shoot and root dry mass at high PO_4^{3-} soil across the concentration gradient. Instead, an increase in shoot and root dry mass compared to the controls and necrosis of leaf tips was observed with increasing As and Sb concentrations (Figure A4.2). These results are supported by Gunes et al. (2009) who reported that PO_4^{3-} may have partially protected chickpea plant growth from inhibition by As, by reducing oxidative stress to cellular membranes. Furthermore, a couple of studies have shown that soybean and sunflower plant biomass increased with PO_4^{3-} addition to As contaminated soils (Azeem et al. 2017, Kamran et al. 2018). In contrast, Cao et al. (2004) showed that the addition of PO_4^{3-} induced As toxicity to carrot and lettuce plant biomass due to increase As accumulation in plant tissues. This suggests that the addition of high PO_4^{3-} concentrations to contaminated soils can ameliorate or mask As toxicity to certain plants such as *B. chinensis* used in this research, however, this is not the case for all plant species.

The chapter 5 showed that only shoot and root dry mass of *B. chinensis* at low PO_4^{3-} soil exhibited a significant dose-response relationship in both As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments (Table 5.2). In regard to individual and combined treatments, the lower EC_{10} and EC_{50} values obtained for shoot dry mass suggested that As in the As $_{(Individual)}$ treatment was more toxic than As + Sb $_{(Combined)}$ treatment. Similarly, for root dry mass, EC_{10} and EC_{50} in As $_{(Individual)}$ treatment was shown at 20 and 40 mg/kg, while As + Sb $_{(Combined)}$ treatment showed EC values of 20 and 56 mg/kg, respectively. However, the confidence intervals of these estimates are overlapping and thus may not be significantly different. In contrast to the results shown here for shoot dry mass, As in As + Sb $_{(Combined)}$ treatment was shown to be more toxic to shoot dry mass of water spinach (*Ipomoea aquatica*) compared to As $_{(Individual)}$ treatment at low PO_4^{3-} soil (Section 4.3.2). Although EC values during the increase of soil PO_4^{3-} have not been extensively studied, Cao et al. (2009) reported that EC_{10} values for As ranged from 79 to 270 mg/kg for wheat and 20 to 156 mg/kg for lettuce. In the same study EC_{50} values (inhibition of root length)

for As have been reported as 159 to 683 mg/kg for wheat and 59 to 426 mg/kg for lettuce (Cao et al. 2009). Thus, it appears that choy sum is sensitive to As contamination.

For Sb, no toxicity was observed in the Sb $_{(Individual)}$ treatment in either the low or high PO_4^{3-} treatments, whereas there was a clear decrease in shoot and root dry mass in the As + Sb $_{(Combined)}$ treatment proportional to the expected toxicity from As (Figure 5.3). Similarly, increased soil PO_4^{3-} had no influence on *B. chinensis* shoot and root lengths in the Sb $_{(Individual)}$ treatment. In support of this, Tschan et al. (2008) also showed that the addition of PO_4^{3-} to Sb contaminated soil had no effect on the growth of maize and sunflower plants.

A slight decrease in shoot lengths from ~100 to 60% was observed within the high PO_4^{3-} treatment within the As + Sb $_{(Combined)}$ treatments, with no clear concentration-response relationship between As and Sb concentrations and effects (Figure A4.3). The lack of toxicity from either the individual As or Sb on shoot length and the subsequent 40% decrease in the combined treatment at the high PO_4^{3-} concentration suggests that the presence of the metalloids together as a mixture can adversely affect the height of *B. chinensis*. When considering the root lengths, increased PO_4^{3-} in soils had no clear influence on *B. chinensis* root length within the As and Sb treatments individually or combined (Figure A4.3). Although studies have looked at the interaction between PO_4^{3-} and As , studies to understand the interaction of PO_4^{3-} on toxicity and bioavailability of Sb individually and in combination with As are still lacking.

Table 5.2. Toxicity of As $_{(Individual)}$ and As + Sb $_{(Combined)}$ exposures to *Brassica chinensis var. parachinensis*. Tabled are concentrations that cause a 10% and 50% (EC_{10} and EC_{50} of SEP-bioavailable As in mg/kg, with 95% confidence intervals) reduction in shoot and root dry mass at low soil PO_4^{3-} concentrations after 40 days of bioassay. No toxicity was observed in the high PO_4^{3-} treatment or Sb $_{(Individual)}$ treatments (data not shown).

	As $_{(Individual)}$	**As + Sb $_{(Combined)}$**
Shoot dry mass		
EC_{10}	19 (2-36)	49 (15-85)
EC_{50}	42 (21-63)	72 (53-91)
Root dry mass		
EC_{10}	20 (0-40)	20 (0-47)
EC_{50}	40 (17-62)	56 (24-88)

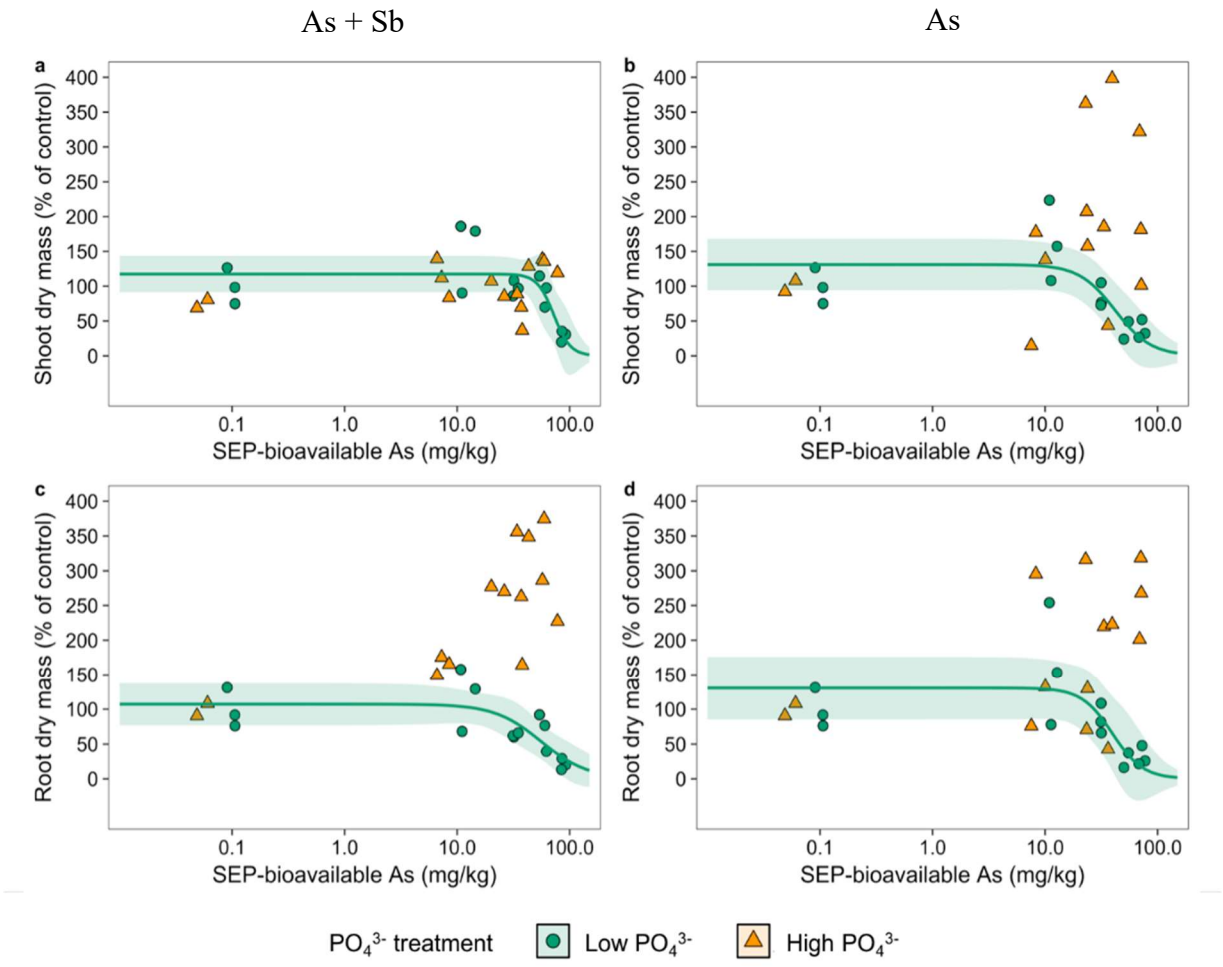


Figure 5.2. The effect of SEP-bioavailable As to *Brassica chinensis* var. *parachinensis*: a, b). shoot dry mass and c, d). root dry mass in As + Sb (Combined) and As (Individual) treatments at low and high PO_4^{3-} concentrations, respectively. No toxicity to shoot or root dry mass was observed in the high PO_4^{3-} concentrations (orange triangles). The fitted line represents a 3-parameter log-logistic model with its 95% confidence interval represented as the surrounding ribbon.

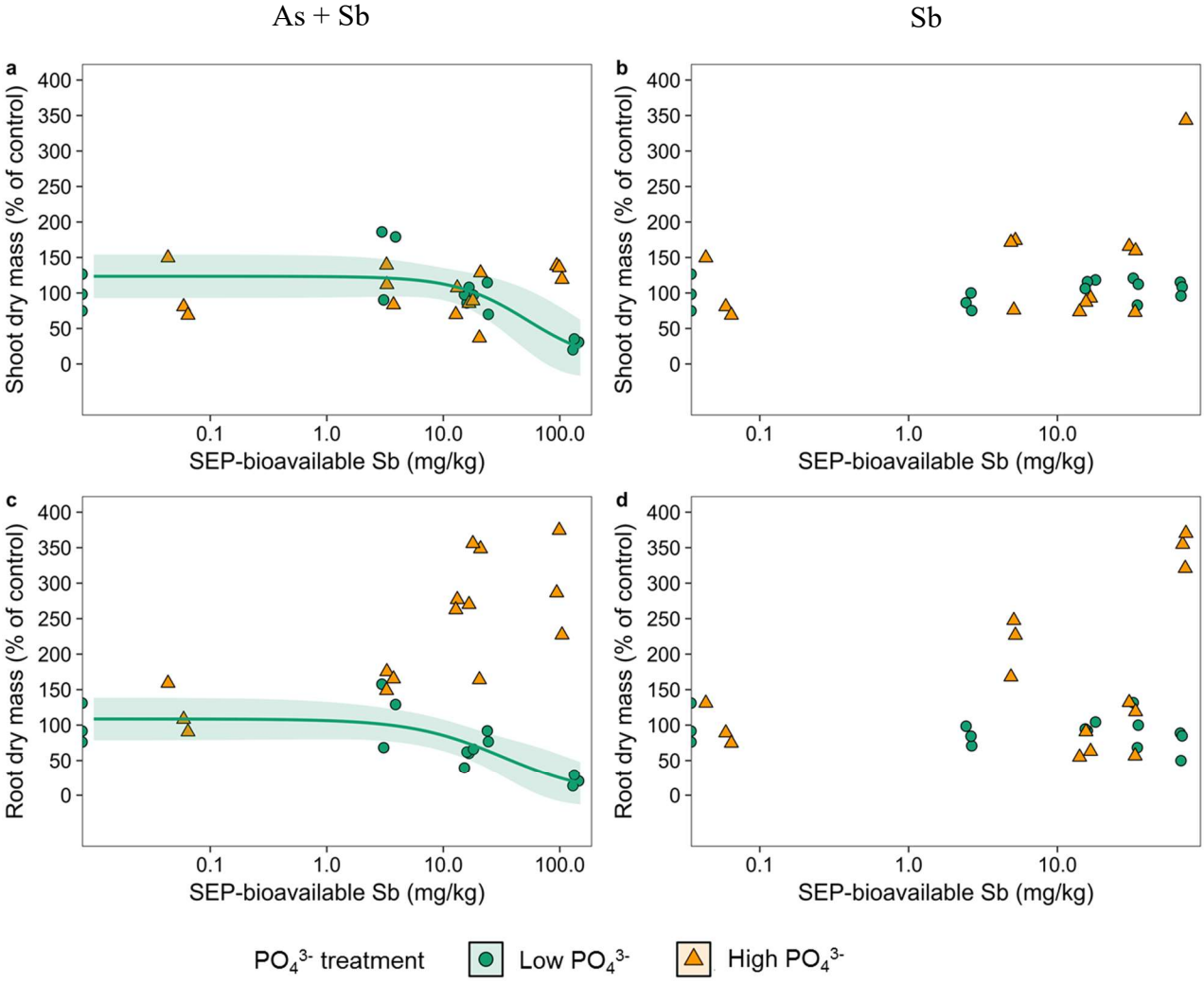


Figure 5.3. The effect of SEP-bioavailable Sb to *Brassica chinensis* var. *parachinensis*: a, b). shoot dry mass and c, d). root dry mass in As + Sb (Combined) and treatments at low and high PO_4^{3-} concentrations. No toxicity to shoot or root dry mass was observed in the high PO_4^{3-} concentrations (orange triangles) or in Sb (Individual) treatments (b, d). The fitted line represents a 3-parameter log-logistic model with its 95% confidence interval represented as the surrounding ribbon.

5.3.4 The effect of PO_4^{3-} addition to As and Sb accumulation

At low PO_4^{3-} soil, As concentrations in *B. chinensis* shoots increased with increasing soil concentrations in both As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments (Figure 5.4a). However, no difference in As accumulation was observed between individual and combined treatments. In high PO_4^{3-} soil, shoot As concentration increased with increasing soil concentrations in As $_{(Individual)}$ treatment whereas shoot As concentration peaked at 47 mg/kg (treatment C3, Figure 5.4a), and then decreased in the highest concentration within the As + Sb $_{(Combined)}$ treatment. On the other hand, root As concentrations in plants grown in high PO_4^{3-} soil were significantly higher than low PO_4^{3-} soil in As $_{(Individual)}$ treatment while the As + Sb $_{(Combined)}$ treatment showed only a slight increase (Figure 5.4b). In As $_{(Individual)}$ treatment, a peak was observed at the second highest concentration (I3) followed by a decrease at the highest soil concentration (I4) in the high PO_4^{3-} soil. However, high As concentrations in roots may be due to adhered soil particles which were retained despite thorough washing prior to drying and digestion. Few studies showed that addition of PO_4^{3-} to the soil contaminated with single-As increased As accumulation in plants; i.e., wheat root and shoot and chickpea shoot (Tao et al. 2006, Gunes et al. 2009, Pigna et al. 2009). This is mainly due to the increase of bioavailable As from the replacement of AsO_4^{3-} by PO_4^{3-} on soil binding sites. Further, this was supported by Cao et al. (2004) who reported that excessive PO_4^{3-} increased As accumulation in carrot (4.56-9.3 times) and lettuce (2.45-10.1 times) due to increased water soluble As.

The bioaccumulation factor (BAF) is used to determine the transport of metalloids from soil to plant root and is based on the concentration ratio in soil and root. For As $_{(Individual)}$ treatment, the BAF of As in *B. chinensis* roots in low and at high PO_4^{3-} ranged from 0.04-0.09 and 0.04-0.14, respectively (Figure 5.4c). For As + Sb $_{(Combined)}$ treatment, the BAF of As in *B. chinensis* roots in low and at high PO_4^{3-} ranged from 0.04-0.07 and 0.07-0.20, respectively. In general, BAF of As at high PO_4^{3-} soil was higher than low PO_4^{3-} soil for most of the concentrations. This suggests that As transport from soil to root was more effective when increasing PO_4^{3-} concentration in soil. In support of this, some studies reported that both AsO_4^{3-} and PO_4^{3-} are taken up by the same transporter system in plant roots; i.e inorganic phosphate transporters (PHT) (Chen et al. 2002, Finnegan et al. 2012, Li et al. 2016).

The translocation factor (TF) is a way to investigate the ability of metalloids transfer from plant roots to shoots (Ngo et al. 2016). In the As $_{(Individual)}$ treatment, there was a lower translocation of As from roots to shoots in high PO_4^{3-} soil (0.03-0.1) compared to low PO_4^{3-} soil (0.2-0.4) (Figure 5.4d). In the As + Sb $_{(Combined)}$ treatment, results were more varied with no obvious trend in soil treatments or between high and low PO_4^{3-} soils. Similar to the results of As $_{(Individual)}$ treatment in Chapter 5, studies by Pigna et al. (2010) and Pigna et al. (2009) showed that As accumulation in wheat roots rapidly increased compared to shoots at excessive PO_4^{3-} concentrations with lower translocation from roots to shoots. The lower translocation of As shoots at high PO_4^{3-} soil may be due to the reduction of AsO_4^{3-} to AsO_3^{3-} and subsequent vascular sequestration in the roots as As(III)–PC complex (Wang et al. 2015). Therefore, in the higher PO_4^{3-} soil more As may be encapsulated in the roots and not transferred to the shoots.

Similar to As, Sb concentrations in *B. chinensis* shoots increased with increasing soil concentrations, regardless of the PO_4^{3-} concentration in soil (Figure 5.5a). In general, Sb accumulation in shoot was higher at high PO_4^{3-} soil than low PO_4^{3-} soil within all Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments except the C4 treatment (Figure 5.5a). Moreover, Sb accumulation in *B. chinensis* roots increased with increasing soil concentrations at all concentrations (Figure 5.5b). There was a higher Sb uptake by roots at high PO_4^{3-} soil compared to low PO_4^{3-} soil in the Sb $_{(Individual)}$ treatment; however, the opposite was observed in the As + Sb $_{(Combined)}$ treatment (Figure 5.5b). Thus, these results suggest that increase of PO_4^{3-} concentration in soil may increase Sb accumulation in *B. chinensis* shoots. Feng et al. (2013) reported that $Sb(OH)_6^-$ uptake did not appear to occur via phosphate pathways and Tschan et al. (2008) showed that the addition of PO_4^{3-} did not affect the uptake of Sb in maize and sunflower plants.

The BAF of Sb in Sb $_{(Individual)}$ treatment in low and high PO_4^{3-} soils ranged from 0.07-0.14 and 0.6-0.8, respectively (Figure 5.5c) whereas in As + Sb $_{(Combined)}$ treatment, the BAF of Sb at low and high PO_4^{3-} soils ranged from 1.2-3.0 and 0.8-1.4, respectively. The lower BAF of Sb at low PO_4^{3-} soil in Sb $_{(Individual)}$ treatment suggests that Sb accumulation from soil to roots was more efficient at high PO_4^{3-} concentrations in soil. However, the opposite was observed in the As + Sb $_{(Combined)}$ treatment. When considering the plant uptake mechanism of Sb, there is no evidence to show that $Sb(OH)_6^-$ and PO_4^{3-} are taken up by the same transporter system in plant roots. In support of this, some studies showed that

Sb(V) uptake is primarily associated with apoplastic pathway which depends on the concentration gradient of ions between plant root and soils solution (Feng et al. 2013, Ji et al. 2018). Additionally, the BAF for Sb showed that As + Sb $_{(Combined)}$ treatments had higher accumulation from soil to roots compared to Sb $_{(Individual)}$ treatment in both low and high soil PO_4^{3-} concentrations. This could be due to high bioavailability of Sb in the combined treatment.

The TF showed that there was no clear difference in Sb translocation from roots to shoots in either the Sb $_{(Individual)}$ or As + Sb $_{(Combined)}$ treatments between low and high soil PO_4^{3-} (Figure 5.5d). This suggests that translocation of Sb from roots to shoots was not affected by the increase PO_4^{3-} concentrations in soils. Currently there is no evidence to suggest that PO_4^{3-} and $Sb(OH)_6^-$ uptake occurs through the same transporters in roots due to their different chemical structure and size (Tschan et al. 2008, Tisarum et al. 2015). Further study is needed to identify the uptake mechanisms of $Sb(OH)_6^-$ in plants.

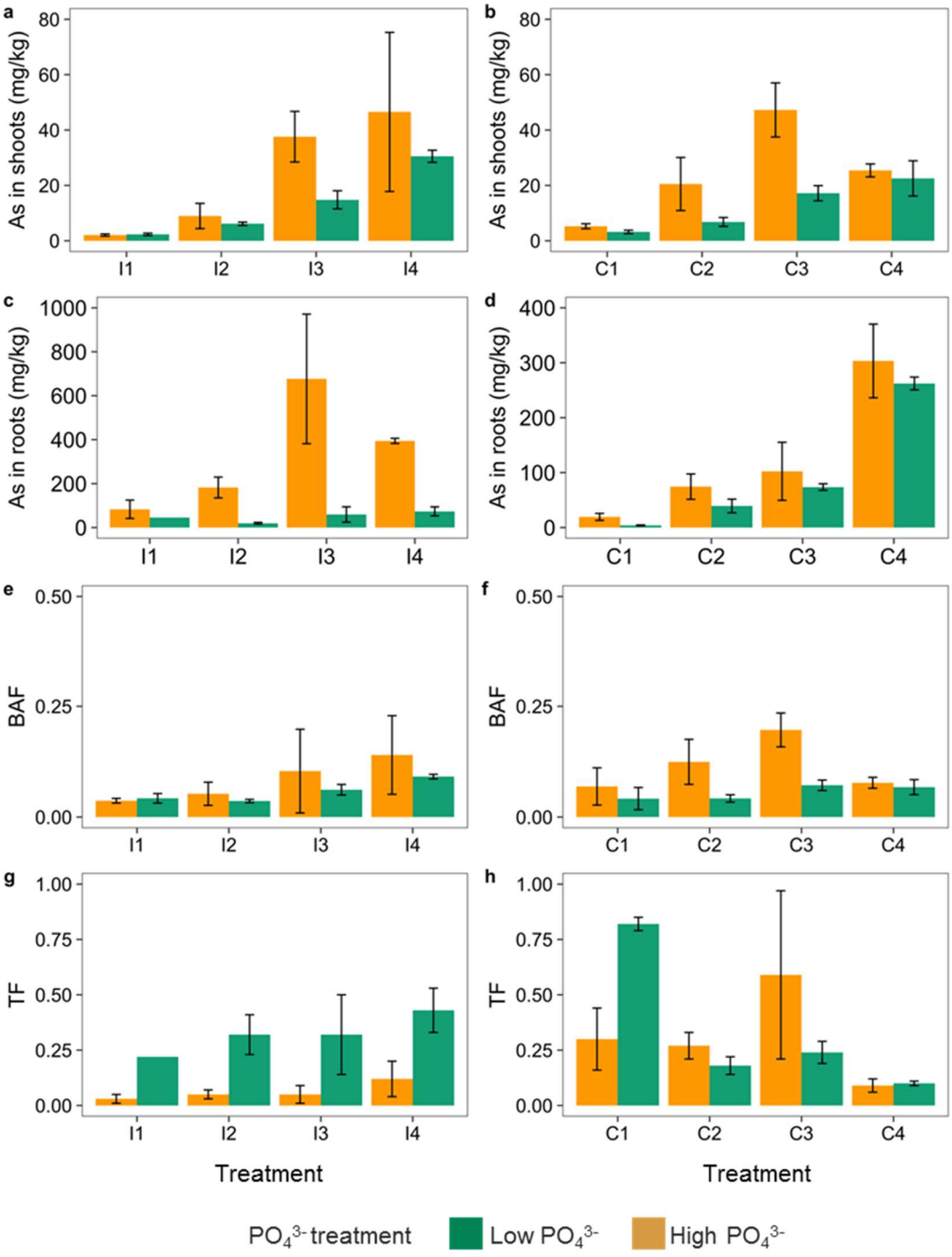


Figure 5.4. The influence of soil PO_4^{3-} on shoot (a and b) and root (c and d) As accumulation in *Brassica chinensis* var. *parachinensis*, including bioaccumulation factors (BAF, e and f) and translocation factors (TF, g and h) for individual and combined treatments, respectively (mean ± SD, n ≤ 3). Individual As concentrations (labelled I1 – I4) and combined As + Sb concentrations (labelled C1 – C4) are given in Table 5.1. Note the different y-axis scales in each graph.

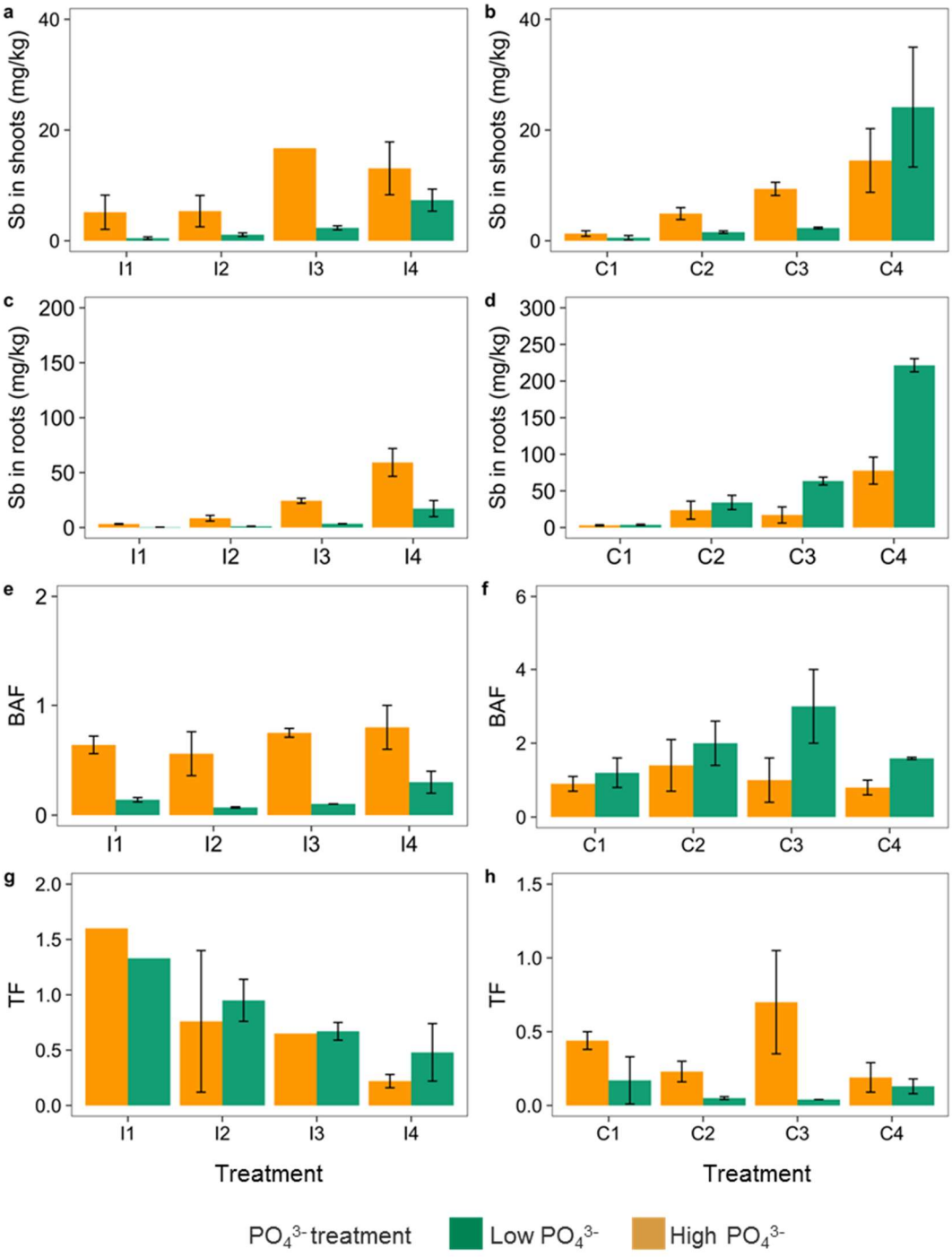


Figure 5.5. The influence of soil PO_4^{3-} on shoot (a and b) and root (c and d) Sb accumulation in *Brassica chinensis* var. *parachinensis*, including bioaccumulation factors (BAF, e and f) and translocation factors (TF, g and h) for individual and combined treatments, respectively (mean ± SD, n ≤ 3). Individual Sb concentrations (labelled I1 – I4) and combined As + Sb concentrations (labelled C1 – C4) are given in Table 5.1. Note the different y-axis scales in each graph.

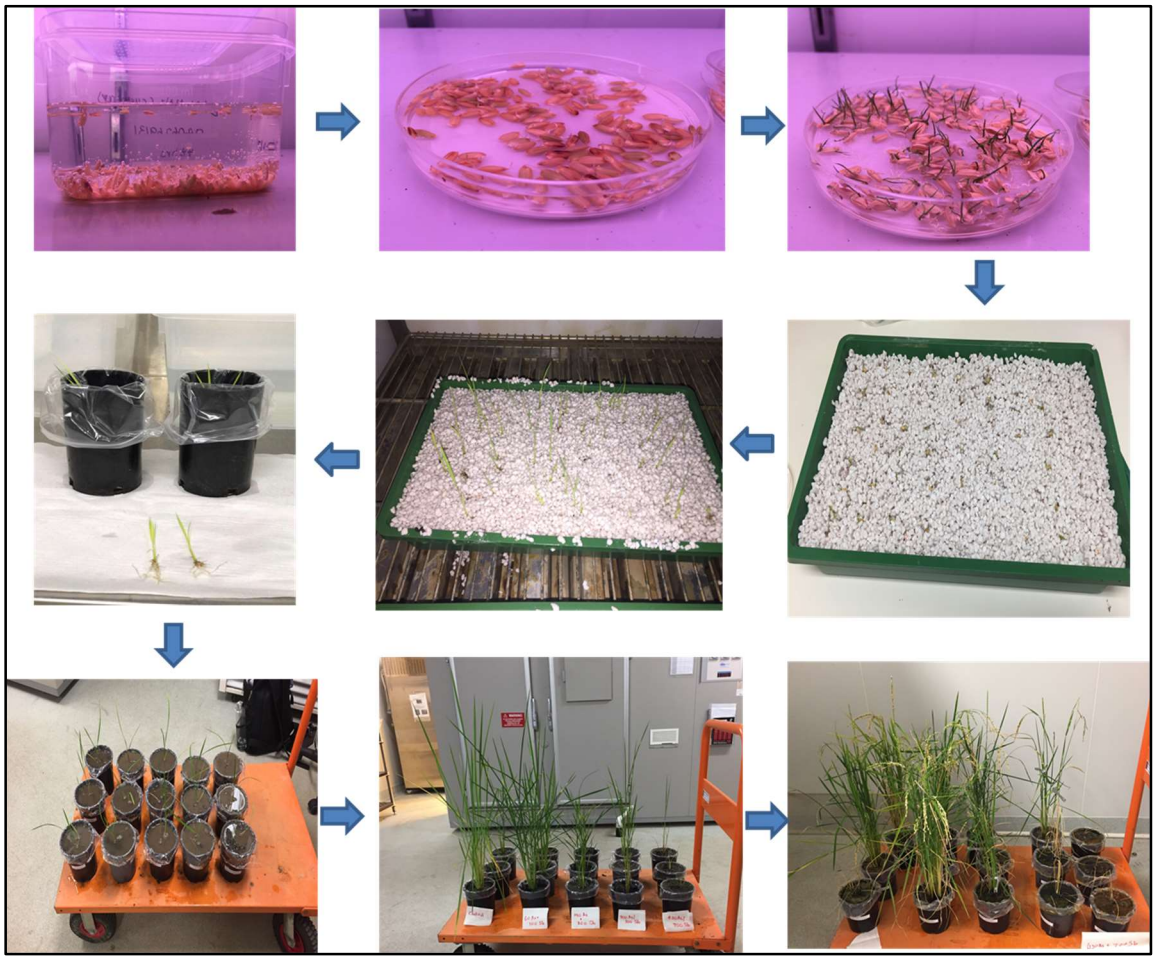
5.4 Conclusion

This chapter investigated the impacts of increasing soil PO_4^{3-} concentrations on As and Sb bioavailability, accumulation and toxicity to *B. chinensis,* particularly under environmentally realistic scenarios of As and Sb co-contamination. Increased PO_4^{3-} in individually contaminated soils had no impact on metalloid bioavailability. Arsenic and Sb in co-contaminated soil had no impact on bioavailability of one another at lower concentrations ($\leq$ 240 mg As/kg and $\leq$ 1680 mg Sb/kg, respectively). However, decreased bioavailability of As and Sb were observed at high PO_4^{3-} concentrations in the soil. Increased PO_4^{3-} in individually or co-contaminated soil increased the accumulation of both As and Sb in the edible parts of *B. chinensis*, except for the highest As and Sb concentrations in the co-contaminated soil.

Arsenic was toxic at low soil PO_4^{3-} concentrations, but no toxicity was observed at high soil PO_4^{3-} concentrations. This is despite more As being accumulated at high PO_4^{3-} concentrations. No toxicity was observed in Sb individually contaminated soil within either low or high PO_4^{3-} treatments. This chapter showed increased soil PO_4^{3-} concentrations ameliorated As toxicity to plant growth although the plant roots and edible parts (shoots) accumulated higher As concentrations.

Increased soil PO_4^{3-} increased As and Sb accumulation in the edible parts of *B. chinensis*, despite having reduced soil bioavailability in soils. A different agricultural practice known to alter soil chemistry, and bioavailability, is waterlogging.

Chapter 6. Assessment of As and Sb bioavailability, accumulation and toxicity to *Oryza sativa l.* cultivated in individually and co-contaminated soils, under waterlogged conditions.



6.1 Introduction

Environmental conditions such as soil flooding or waterlogging will drive soils to anoxia due to the consumption of available oxygen/oxic species. This lowers the soil redox potential and changes metalloid speciation, which can change the behaviour of metalloids in soils. Different As and Sb species have different binding affinities to soil sorption sites including Fe, Al and Mn (hydr)oxides (Section 1.3). For example, under aerobic conditions, As(V) and Sb(V) bind strongly to Fe (hydr)oxides decreasing their mobility in soil (Section 1.3.3) whereas under anaerobic conditions, As(III) and Sb(III) become more mobile due release from Fe (hydr)oxides as a result of reductive dissolution (Ritchie et al. 2013, Hockmann et al. 2014). Thus, it is possible that waterlogging conditions in contaminated agricultural lands, affects As and Sb bioavailability, accumulation and toxicity to agricultural plants.

The various As and Sb species have different uptake mechanisms in plants. Arsenic (V) uptake occurs through the phosphate transport system in plant roots due to similar coordination geometries between AsO_4^{3-} and PO_4^{3-} (Gunes et al. 2009, Pigna et al. 2010, Anawar et al. 2018). Antimony (V) forms octahedral coordination geometry and is assumed to taken up through apoplastic pathway (Tschan et al. 2008, Mathews et al. 2011, Feng et al. 2013). Under anaerobic conditions, As(III) and Sb(III) form similar tetrahedral geometries, and it is suggested that they enter plant roots via similar aquaporin transporters (Ji et al. 2018). This may be a particular concern for plants grown in anaerobic waterlogged conditions. The release of O_2 from the rice rhizosphere can precipitate Fe (hydr)oxide on the root surface forming Fe plaque (Liu et al. 2008). The sequestering of As and Sb by Fe plaque which leads to decrease in plant accumulation has been well studied in individual metalloid exposures (Xie et al. 1998, Abedin et al. 2002, Meharg et al. 2003, Okkenhaug et al. 2012, Ren et al. 2014). However, little is known about how As and Sb co-contamination affects plant uptake, tissue distribution and toxicity in co-contaminated soils under anaerobic conditions.

Rice is the major food crop for half of the world's population, mainly in Asian countries, such as Bangladesh, West Bengal, China, Taiwan and Thailand, which also have elevated As and Sb concentrations in soils and groundwater (Meharg 2004, Okkenhaug et al. 2012, Wu et al. 2019). Moreover, rice (*Oryza sativa*) is generally grown in flooded soils where

As and Sb mobility in soils and bioavailability to plants is likely to be enhanced by reductive dissolution. Thus, more information is needed regarding the As and Sb bioavailability, accumulation and toxicity in soil-rice plant systems.

This chapter investigated the behaviour of As and Sb in the soil-rice plant system across its whole growth period using a rice bioassay grown in individually and co-contaminated soils. The objectives are to: 1) study the individual and combined phytotoxic effects of As and Sb on the growth of rice by comparing the bioavailable concentrations of As and Sb with growth-based endpoints such as dry mass of roots, shoots and grains and lengths of roots and shoots; and 2) determine the competitive effect of As and Sb on accumulation and translocation of these metalloids from soil to different tissues of *Oryza sativa*. This will provide important information about the impact of As and Sb co-contaminated soils on rice crops.

6.2 Methods

6.2.1 Soil preparation

The preparation of soils is outlined in Section 2.2. The uncontaminated soils were mixed (thoroughly in a cement mixture) in a 1:1 ratio with "all-purpose garden soil" (Richgro All Purpose Garden Soil Mix from Bunnings, Australia) to achieve nutritional characteristics for healthy plant.

Arsenic and Sb solutions spiked (as per Section 2.2) to achieve a gradient of four concentrations of: $As_{(Individual)}$ 60, 180, 300 and 420 mg/kg; $Sb_{(Individual)}$ 100, 300, 500 and 700 mg /kg (herein these individually contaminated soils are referred to as I1 to I4); and $As + Sb_{(Combined)}$ 60 + 100, 180 + 300, 300 + 500, 420 + 700 mg/kg (herein the co-contaminated soils are referred to as C1 to C4). The nominal concentrations used in this research are environmentally relevant and reflect concentrations reported for contaminated paddy soils in the literature (Okkenhaug et al. 2012). The soils were equilibrated in a greenhouse for 2 weeks prior to commencement of bioassays to establish soils reflecting a recent contamination event.

6.2.2 Soil characterisation

Soil samples were collected after equilibration and stored in plastic bags for subsequent analyses. The physical and chemical properties; soil pH, particle size, soil moisture, TOC, TKN and extractable phosphorus were measured as described in Section 0.

6.2.3 Preparation of *Oryza sativa* seedlings and establishing the bioassay

Rice seeds were purchased from Herbalistics as outlined in Section 2.3. The rice seeds were sterilized in 30% H_2O_2 (w/w) solution for 15 min and thoroughly rinsed with high purity water. Then the seeds were soaked in high purity water, in a sterilized container, for 48 h (25°C), germinated on a moistened filter paper and placed in a petri dish under ambient greenhouse conditions (Ren et al. 2014). After germination, they were transferred to moist perlite (Huang et al. 2012). At the one-leaf stage, the seedlings were treated with a nutrient solution, as per Yoshida et al. (1971), and grown to a three-leaf stage.

The treatment pots were prepared in triplicate per concentration by adding ~ 2.5 kg of equilibrated soil to circular plastic pots with dimensions of 13 cm (diameter) x 20 cm (height). The pots were lined with a high-density polyethylene bag to maintain waterlogged conditions. After germination, healthy seedlings were transferred to pots (one seedling per pot), this marked the beginning of the bioassay. Pots were placed in a growth chamber under controlled conditions as described in Section 2.3.1. The temperature was 20°C (night) and 28°C (day) and relative humidity maintained at 60-70%. Nutrients were added in the forms of nitrogen ($CO(NH_2)_2$, 0.15 g/kg), phosphorus ($NH_4H_2PO_4$, 0.1 g/kg) and potassium (K_2SO_4, 0.04 g /kg) as a top dressing every 2 weeks.

After ~100 days, bioassays were terminated and seeds (grains plus panicles) were harvested. The remaining rice plants including roots and shoots were separated as per Section 2.3.2 and plant tissue was collected for As and Sb analysis.

6.2.4 Determination of *Oryza sativa* growth

Root, shoot and grain dry mass, root and shoot lengths, and the number of tillers and panicles per plant were recorded as the biological endpoints in determining toxic effects to plant growth. All measurements except the number of tillers were recorded after ~100 days of transplantation (after harvesting). The number of tillers per plant (per pot) were

measured at the maximum tillering stage (after ~60 days of transplantation). The shoots, roots and grains were oven-dried at 70°C for 72 h and the dry masses recorded. Panicles were removed before measuring the shoot dry mass per pot. Grains were separated from the panicle by hand and the grain dry mass per plant was determined. The number of dead plants was also counted at the end of the bioassay.

6.2.5 Analysis of As and Sb concentrations

The sequential extraction procedure (SEP) was derived from Wenzel et al. (2001) as outlined in Section 2.5.1. The total As and Sb concentrations in soil and plant samples were extracted and determined as per Section 2.5.2 and 2.5.3, respectively.

All digests and SEP-extracts were analysed by Thermo iCAP-Q quadrupole ICP-MS as described in Section 2.6. Method detection limits for plant digestions were 0.064 mg As/kg and 0.077 mg Sb/kg and for soil digestions were 2.622 mg As/kg and 0.029 mg Sb/kg. Soils CRM recoveries were within 85-105% and 83-90% of expected values for As and Sb in soils, respectively, and within 96-130% and 80-84% of expected values for As and Sb in plants, respectively.

6.2.6 Data analysis

All data are presented as mean ± SD $n \leq 3$ (3 pots per treatment, 1 plants per pot) and were performed as per Section 2.7. Dead plants were included in dose-response curves with their endpoints reported as 0. Interactive effects of the combined treatment were investigated using a toxic units (TU) approach as presented in Equation 4.2. The bioaccumulation factor (BAF) and translocation factor (TF) were determined as per Equation 5.1 and Equation 5.2, respectively.

6.3 Results and discussion

6.3.1 Physical and chemical properties of soil

The physical and chemical characteristics of the studied soil are shown in Table 6.1. In general, all test soils (control, $As_{(Individual)}$, $Sb_{(Individual)}$ and $As + Sb_{(Combined)}$) were silty-sand and near-neutral pH, with no differences in characteristics between the control and contaminated soils used in this research. These results confirm that soil characteristics did not contribute to the adverse effects observed in plant physiology during bioassays.

The total As and Sb concentrations in soils were determined before the start of the bioassay (**Table 6.2**). The total concentrations measured are significantly lower than the nominal concentrations and this may be due to unbalance (lumpy) distribution of metalloids occurring during addition of metalloid solution to the soil and leaching during equilibration time. However, all data analysis was done on the actual measured concentrations. The total As concentration in soil ranged from 31-130 and 32-116 mg/kg for the $As_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments, respectively. For Sb, the total Sb concentration in soil ranged between 30-277 and 31-208 mg/kg for the $Sb_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments, respectively. The As and Sb concentrations in uncontaminated soils usually range from 1-40 mg/kg (Herath et al. 2016) and 0.3-8.6 mg/kg, respectively (Tschan et al. 2009). Arsenic and Sb concentrations in paddy soil near Sb mining sites have been reported to be as high as ~530 mg/kg and ~1560 mg/kg, respectively (Okkenhaug et al. 2012, Lin et al. 2015). Thus, the concentrations used in this research are representative of contaminated paddy soils.

6.3.2 Different fractions of As and Sb

The association of As and Sb with different soil solid phases in the test soils are shown in Figure 6.1. The SEP- extractable As and Sb concentrations in soils were determined before the start of the bioassay (Table 6.2). In general, the total SEP-extractable As in both $As_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments increased with increasing concentrations in soil. The total SEP-extractable fraction includes As and Sb found in soluble (non-specifically bound) phase, exchangeable (specifically bound) phase, bound to amorphous (non-crystalline) and crystalline Fe/Al soil phases.

Table 6.1. The physical and chemical characteristics of soils.

Soil characteristics	Contaminated soil	Control soil
Sand, (>62.5 µm –2 mm) (%)	53 ± 2	52 ± 1
Silt (>25–62.5 µm) (%)	44 ± 2	44 ± 1
Clay (3.9–25 µm) (%)	3.4 ± 0.2	3.73 ± 0.08
pH	6.9 ± 0.1	6.8
Total Organic Carbon (TOC, %)	11 ± 1	11*
Total Kjeldhal nitrogen (TKN, mg/kg)	400 ± 30	440 ± 60
Phosphorous content (mg/kg)	40 ± 5	40*
Total moisture content (%)	4.4 ± 0.2	1.7*

* Only one control soil sample was available due to a measurement error.

Arsenic in the As $_{(Individual)}$ treatment was associated with different soil-binding phases in the following order; specifically bound (24-56% of total) > associated with amorphous Fe/Al oxides (19-32% of total) > non-specifically bound (3-11% of total) > associated with crystalline Fe/Al oxides (0.6-6% of total) (Figure 6.1). Similar to the As $_{(Individual)}$ treatment, the As in the As + Sb $_{(Combined)}$ treatment was mainly associated with specifically bound fraction. However, minor differences were observed between the binding phases of the combined treatment compared to individual treatment; specifically bound (25-46% of total) > associated with amorphous Fe/Al oxides (17-34% of total) > non-specifically bound (2-13% of total) > associated with crystalline Fe/Al oxides (0.2-6% of total). Thus, the As was mainly found in soluble and exchangeable forms in the soils used in this research. This may be related to the soluble form of added As and the shorter equilibration time (2 weeks) in the soils as Chapter 3 it was found that the lability of As in soils is reduced with increasing ageing time. Niazi et al. (2011) also showed relatively high As concentrations in non-specifically and specifically bound fractions in spiked soils compared to aged contaminated soils from cattle dip sites and railway corridors.

In the Sb $_{(Individual)}$ treatment, a relatively high proportion of Sb was extracted from amorphous Fe/Al oxides (13-46% of total), while the smallest fraction was extracted from crystalline Fe/Al oxides (1-6% of total) and 7-21% and 4-24% of total Sb were found as soluble and exchangeable forms, respectively. A similar pattern of association was

observed in Sb in the As + Sb $_{(Combined)}$ treatment, with minor differences in some binding phases; associated with amorphous Fe/Al oxides (13-27% of total) > non-specifically bound (9-18% of total) ~ specifically bound (6-18% of total) > associated with crystalline Fe/Al oxides (2-4% of total). Unlike As, a relatively greater proportion of Sb was associated with amorphous Fe/Al oxides leading to lower lability in both individual and combined treatments. In support of this, Tighe et al. (2007) also demonstrated that large proportion of Sb was bound to amorphous Fe/Al oxides using a modified sequential extraction method used for P extraction. This showed that Sb efficiently binds to amorphous oxides over a short equilibration time and also readily available to become more mobile during waterlogging or under anaerobic conditions.

On average, the proportion of As and Sb extracted in the non-specifically bound fraction was slightly increased at higher total soil As and Sb in the co-contaminated compared to individually treatments contaminated soils. This may be due to the saturation of soil sorption sites by the added As and Sb in the soils as proposed in Section 4.3.1. For both As and Sb treatments, bioavailable As and Sb increased with increasing metalloid concentrations (Table 6.1 and Figure A5.1). The presence of both As and Sb in the co-contaminated soils had little to no influence on their SEP-bioavailable fractions compared to the individually contaminated soil.

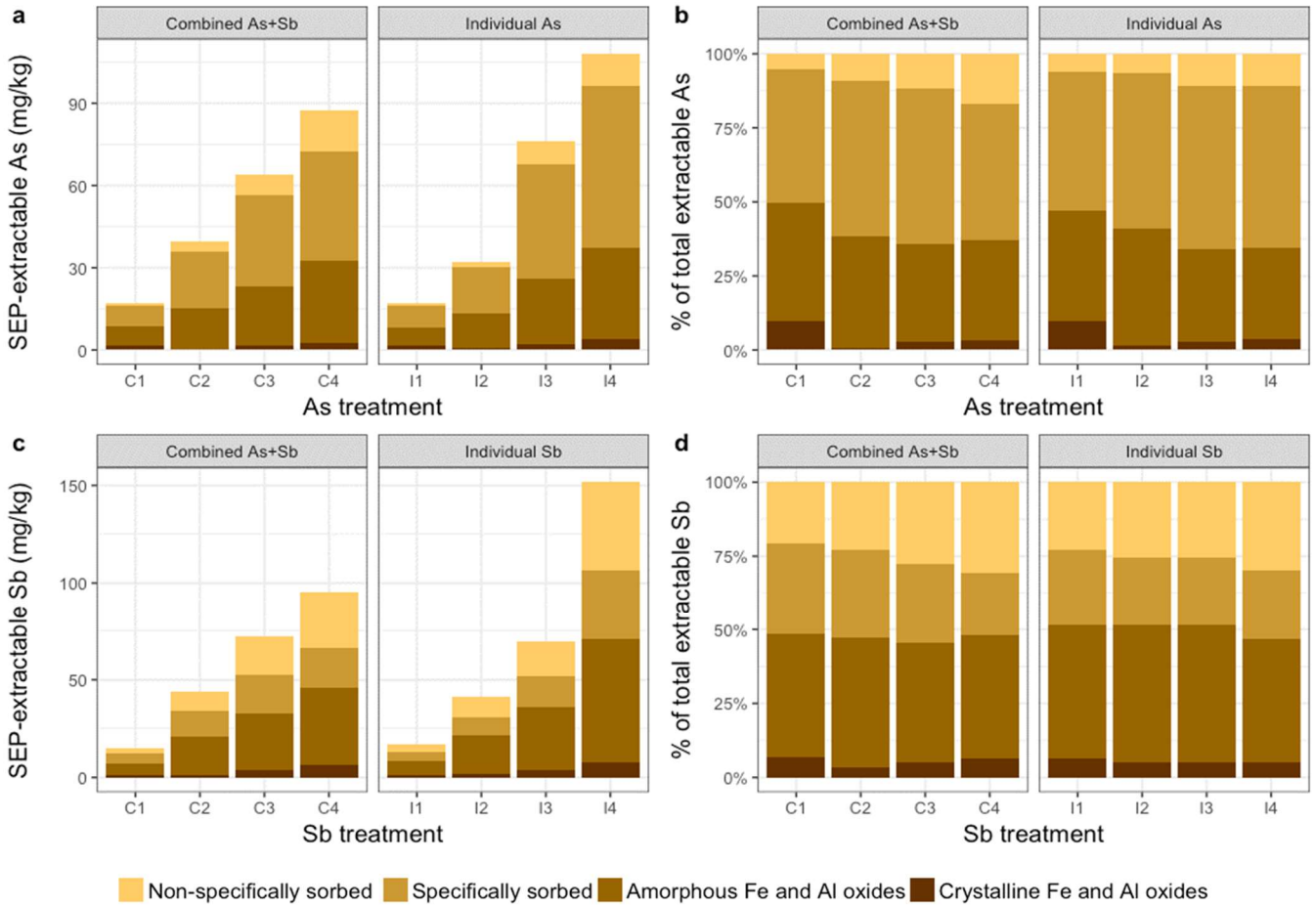


Figure 6.1. Association of As (a, b) and Sb (c, d) with different soil compartments in the bioassay, extracted by sequential extraction procedure as per Wenzel et al. (2001). Measurements are based on dry soil mass (n=3). C1-4 consist of the different metal treatments in the combined (As + Sb $_{(Combined)}$) contaminated soils and I1-4 consist of the different metal treatments in the individually (As $_{(Individual)}$, Sb $_{(Individual)}$) contaminated soils.

Table 6.2. Total soil and SEP-bioavailable concentrations (measured) of As and Sb for As + Sb $_{(Combined)}$, As $_{(Individual)}$ and Sb $_{(Individual)}$ soil treatments (mean ± SD, n ≤ 3).

	Total soil concentration		**SEP-bioavailable**		**Bioavailability of total soil concentration**	
	As (mg/kg)	**Sb (mg/kg)**	**As (mg/kg)**	**Sb (mg/kg)**	**As %**	**Sb%**
Control	8 ± 2	0.48 ± 0.06	0.97 ± 0.06	< 0.029	13 ± 4	< 6.1 ± 0.8
As + Sb $_{(Combined)}$						
C1	32 ± 3	31 ± 1	7 ± 4	7 ± 3	22 ± 12	21 ± 9
C2	55 ± 4	81 ± 3	24 ± 1	23 ± 2	44 ± 3	29 ± 2
C3	81 ± 7	137 ± 9	41 ± 1	39 ± 6	51 ± 5	29 ± 3
C4	116 ± 8	208 ± 11	55 ± 6	50 ± 8	47 ± 4	24 ± 5
As $_{(Individual)}$ and Sb $_{(Individual)}$						
I1	31 ± 3	30.1 ± 0.2	19 ± 1	8 ± 1	30 ± 4	27 ± 3
I2	45 ± 3	87 ± 7	19 ± 1	20 ± 4	42 ± 3	40 ± 6
I3	88 ± 5	143 ± 4	50 ± 1	34 ± 3	57 ± 4	14 ± 3
I4	130 ± 20	277 ± 5	71 ± 2	81 ± 1	57 ± 10	29 ± 1

6.3.3 Toxicity response of As and Sb to *Oryza sativa* L. growth

Tiller number

A tiller is a stem which grows after the parent shoot through germination and is indicative of vegetative growth. *O. sativa* grows most rapidly at the tillering stage (vegetative stage, ~14 days after transplanting) (Liu et al. 2018) and thus, the number of tillers were recorded to determine the effect of soil As and Sb on the rice's vegetative stage. The morphology of *O. sativa* within the As $_{(Individual)}$, Sb $_{(Individual)}$, As + Sb $_{(Combined)}$ treatments after ~ 100 days are shown in Figure 6.2a. Increased As and Sb concentrations, both individually and combined, decreased the *O. sativa* tiller production, affecting vegetative growth. Elevated exposure concentrations reduced the average tiller number by up to 81% compared to the controls, i.e. from 26 in the control treatments to as few as 5 in the highest As treatment (Figure 6.2b) and 10 in the highest Sb treatment.

A decrease in tiller number with increasing As soil concentration has been observed previously (He et al. 1999, Abedin et al. 2002, Azizur Rahman et al. 2007, Shah et al. 2014). A similar reduction in rice yield associated with reduced number of tillers has been observed by Panaullah et al. (2009) at soil As concentrations of ~70 mg/kg. Further, Azizur Rahman et al. (2007) showed that increased As concentrations in soil decreased the photosynthetic pigment content and thereby reduced tillering and shoot biomass. In addition, Shah et al. (2014) reported that increases in As concentrations in irrigation water reduced the number of tillers and panicles and plant heights with greater reduction observed under anaerobic conditions than with aerobic conditions.

A poor dose-response relationship was observed for Sb concentrations and *O. sativa* tiller number in Sb $_{(Individual)}$ treatments due to the variability in the replicates of each concentration. The general trend, however, was that the number of tillers decreased with increasing Sb soil concentrations (Figure A5.2). Within the As + Sb $_{(Combined)}$ treatment, tiller numbers were reduced from 26 to 0 at the highest soil As and Sb concentrations, representing complete inhibition. Effective concentrations (EC) of As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments which decreased tiller number by 10 and 50% are reported in Table 6.3.

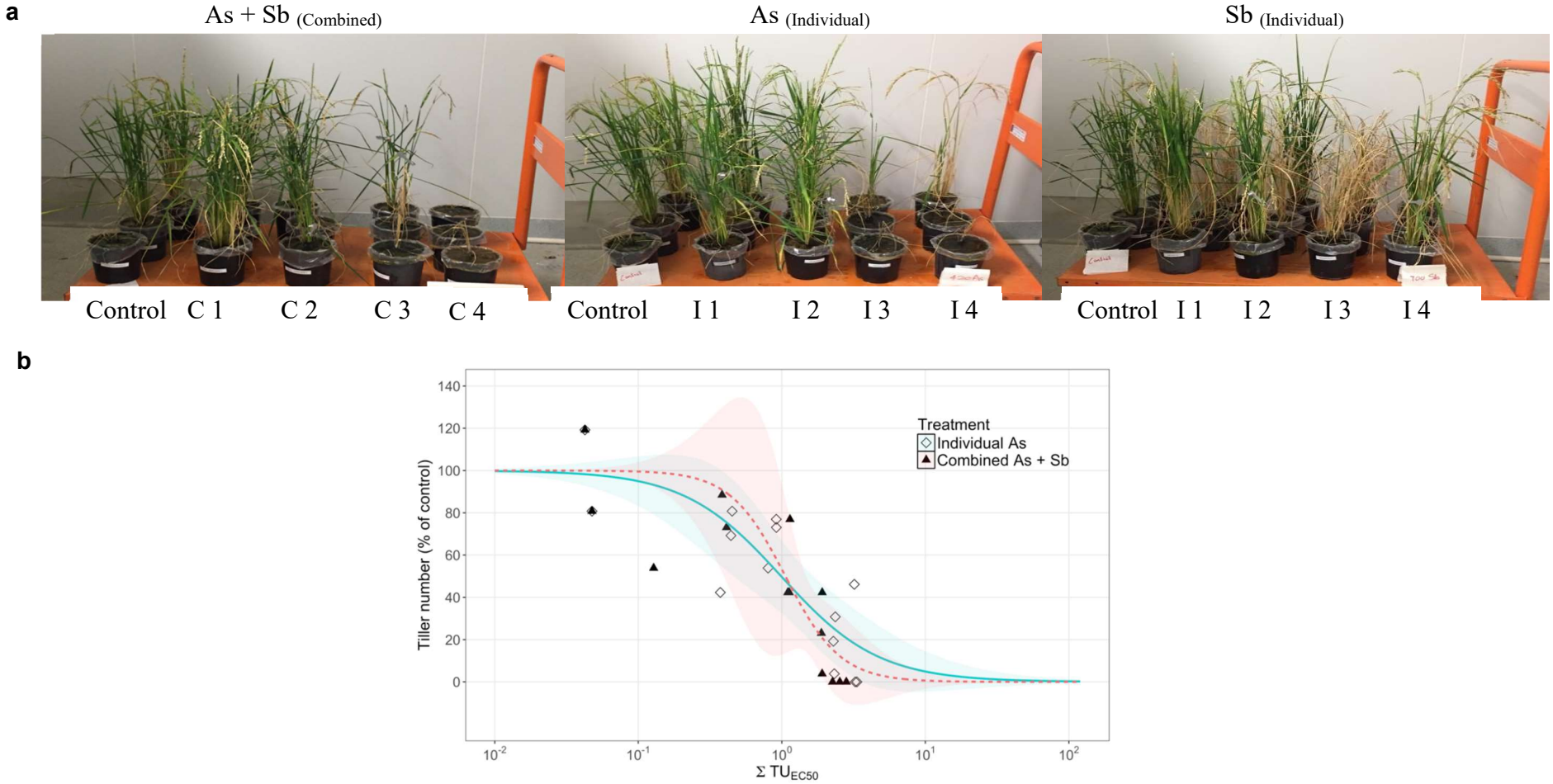


Figure 6.2. The effect of As and Sb on the a). morphology of *Oryza sativa L.* plant grown for ~100 days after transplanting and b). number of tillers per pot at maximum tillering stage (% control) plotted against toxic units (TU) for As (Individual) and As + Sb (Combined) treatments. Due to the poor dose-response relationship in the individual Sb treatment, toxic units could not be calculated for Sb concentrations. The confidence Interval (CI) for As + Sb (Combined) treatments reflects the lack of data around the EC_{10}.

Shoot and root dry mass

Shoot and root biomass of *O. sativa* exhibited a dose-response relationship with concentrations in the As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments while exposure to Sb $_{(Individual)}$ had a poor relationship. In the As $_{(Individual)}$ treatment, two of the three replicate plants died when exposed to the highest concentration, and shoot and root dry mass was reduced to 25% and 6.6% compared to the controls within the I3 treatment (Figure 6.3a and b), respectively. A slight decrease in shoot and root dry mass of *O. sativa* was also observed within Sb $_{(Individual)}$ treatments (Figure A5.3). In the As + Sb $_{(Combined)}$ treatment, all plants died at the highest concentration, indicating that combined As and Sb is acutely toxic to rice at concentrations of 116 mg (As)/kg and 208 mg (Sb)/kg. This toxicity was also evident in the second highest (C3) As+ Sb treatment as shoot and root dry mass significantly reduced to an average of 31% and 17%, respectively, compared to the controls. This indicates that As in both As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments caused a greater reduction to root dry mass compared to shoots.

Individual exposure to As decreased the vegetative phases of *O. sativa* as well as root and shoot dry mass compared to controls. Root dry mass was found to be the most sensitive to As. This was supported in several studies, including Abedin et al. (2002) who showed a reduction in shoot and root dry mass by 36% and 34% of the controls, respectively, at As concentrations of approximately 200 mg/kg. Panaullah et al. (2009) also reported a reduction in shoot dry mass with increasing As concentrations in soils up to ~70 mg/kg. Abedin et al. (2002) also showed that increased As concentrations in irrigation water (ranging from 0-8 mg/L) reduced shoot and root dry mass.

Individual exposure to Sb reduced *O. sativa* plant growth compared to controls by effecting their vegetative and reproductive phases, but no strong concentration-dependent response was observed at the concentrations tested. Further, Sb showed a poor dose-response relationship to root and shoot dry mass, lengths, grain yield, or grain dry mass compared to the controls. Although there are many studies on Sb accumulation in rice, to our knowledge only He et al. (1999) investigated effects of Sb on growth parameters and crop yield of rice. He et al. (1999) observed a decrease in biomass and grain yield of rice by 12% following exposure to Sb(V) and ~44% and ~22%, respectively following exposure to Sb(III) at 300 mg/kg. However, at the highest total soil concentration (1000

mg/kg), the effect of Sb(V) and Sb(III) were much larger (decreased biomass and grain yield of rice by16% and 20% following exposure to Sb(V) and ~65% and ~90%, respectively) with Sb(III) being more toxic than Sb(V).

The EC_{50} for shoot dry mass in the As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments were 25 (14-36) and 26 (18-35) mg/kg, respectively (Table 6.3), however, their 95% CI showed that these EC_{50} values are not significantly different. Similarly, EC_{50} values for root dry mass were not significantly different between As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments (8 (0-17) and 8 (0-18) mg/kg, respectively). A similar pattern was observed for EC_{10} with both treatments. The EC values calculated for root dry mass must be considered carefully due to the large variation in the control-treatment's response. To our knowledge, no previous studies have examined the combined toxicity of As and Sb on the growth of *O. sativa.* The Chapter 4 on *l. aquatica* under aerobic conditions observed a lower EC_{50} value for shoot dry mass in an As + Sb $_{(Combined)}$ treatment (20 (7.4-33) mg/kg) compared to an As $_{(Individual)}$ treatment (81 (56-106) mg/kg). This suggests that the As + Sb $_{(Combined)}$ treatment was more toxic towards shoot dry mass compared to As $_{(individual)}$ treatment for *l. aquatica* but not for *O. sativa.*

Shoot and root lengths

Shoot and root lengths decreased within the As $_{(Individual)}$ treatments to an average of 68% and 61%, respectively compared to controls within the I3 treatment (Figure 6.3cand d, respectively). Similar to the results obtained for shoot and root dry mass, no clear dose response was shown for shoot and root lengths when exposed to Sb concentrations in the soil; however, shoot and root lengths were decreased at the highest bioavailable Sb concentration (I4). The combination of As + Sb significantly reduced shoot and root lengths to an average of 76% and 66% of their controls, respectively, within the second highest concentrations (C3 treatment).

For shoot lengths, the EC_{50} values were 60 (50-69) and 42 (41-43) mg/kg for As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments, respectively. This showed that As + Sb $_{(Combined)}$ treatment was more toxic to shoot elongation compared to the As $_{(Individual)}$ treatment. Similarly, a lower EC_{50} (42 (39-44) mg/kg) was obtained for root lengths in the As + Sb $_{(Combined)}$ treatment, showing that As and Sb co-contamination was more toxic compared to the individual contamination of As and Sb (EC_{50}: 59 (45-72) mg/kg, Table 6.3). In

contrast, as outlined in Chapter 4 *I. aquatica* showed no significant difference in the EC_{50} values for shoot lengths between As $_{(Individual)}$ (65 (38-94) mg/kg) and As + Sb $_{(Combined)}$ (72 (43-101) mg/kg) treatments (Section 4.3.2).

Panicle number, grain mass and grain yield

The reproductive growth phase starts with panicle formation, which bears the rice spikelets. This research showed a reduction in the number of panicles following exposure to As and Sb in all treatments (Figure 6.3). The number of *O. sativa* panicles was significantly reduced to 16% (relative to controls) within the second highest I3 As $_{(Individual)}$ treatment (Figure 6.3e). In the As + Sb $_{(Combined)}$ treatment no panicles were observed at the highest As and Sb concentrations (C4) due to plant death, with the second highest C3 treatment reducing panicle number by 6% compared to controls. Antimony exposure also reduced panicle number but the dose-response relationship was unable to derive due to high variability in the response (Figure A5.2). Further testing is needed to resolve this.

The rice grain dry mass per pot decreased with increasing As and Sb concentrations in both individual and combined exposures, but dose-response relationships were observed only in the As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments (Figure 6.3f). In the As $_{(Individual)}$ treatment, grain yield was reduced to 8% of the controls at the highest soil As concentration (I4) and to 3% within the second highest C3 combined treatment. A similar reduction in the number of panicles has been observed by Panaullah et al. (2009) and Azizur Rahman et al. (2007) with increasing As concentrations in soil. Shah et al. (2014) reported that increased As concentrations in irrigation water reduced the number of rice panicles to a greater extent under anaerobic conditions compared aerobic conditions.

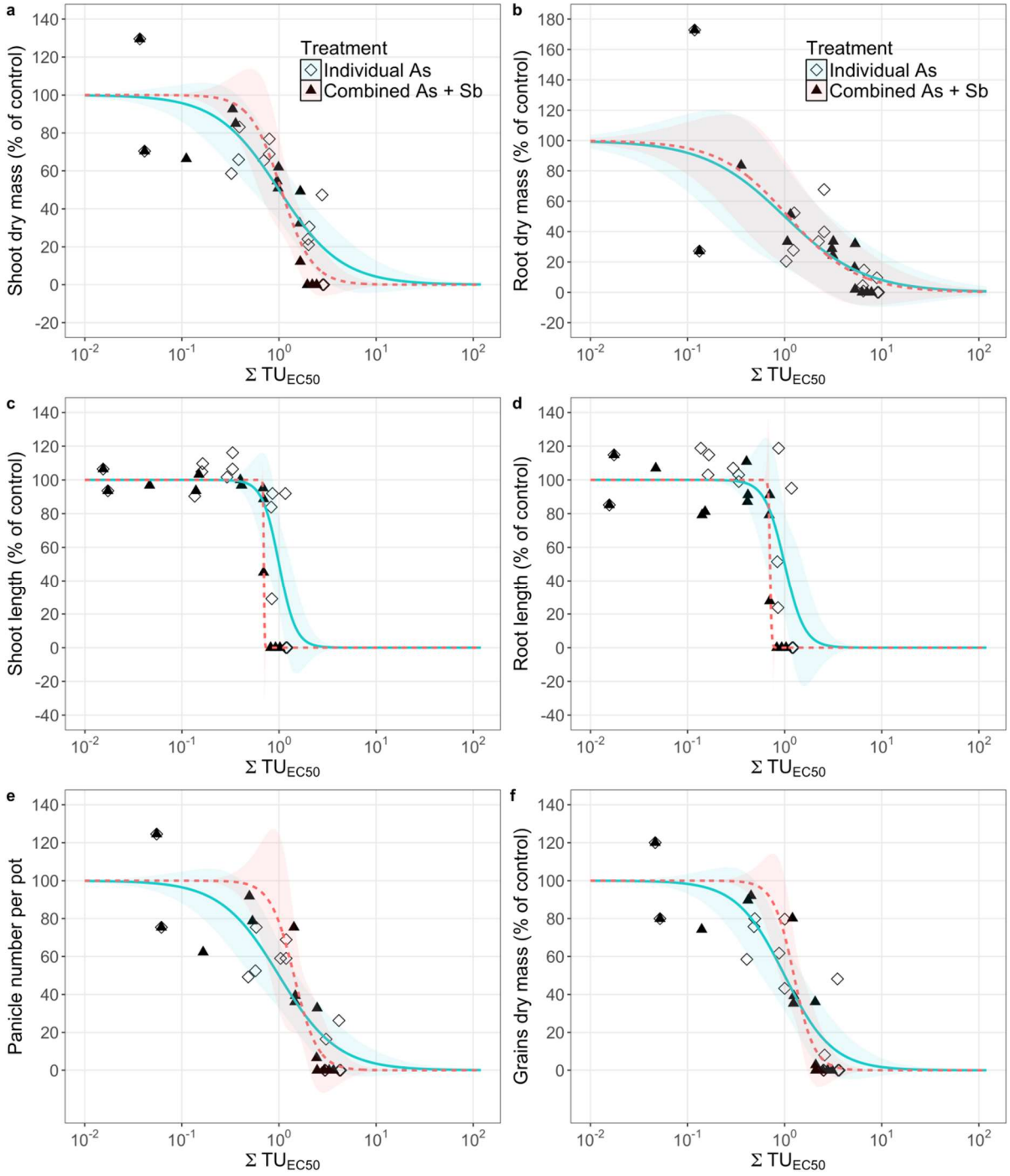


Figure 6.3. The relationship between As and As + Sb on a). shoot dry mass, b). root dry mass, c). shoot length, d). root length, e.) panicle number, and f). grain dry mass (% of control) of *Oryza sativa L* and toxic units (TU) in the As (Individual) and As + Sb (Combined) treatment. Due to the poor dose-response relationship of these effects with Sb in the individual treatment, toxic units were calculated only with As concentrations (effects with Sb are shown in (Figure A5.2 and Figure A5.3)). The confidence Interval (CI) for figures e and f reflects the lack of data around the EC_{10}.

Table 6.3. Toxicity of As in individual (As $_{(Individual)}$) and combined (As + Sb $_{(Combined)}$) exposures to *Oryza sativa L.* with concentrations that cause a 10% and 50% (EC_{10} and EC_{50}, with 95% confidence intervals (CI)) toxic effect to endpoints. Concentrations reported are SEP-bioavailable concentrations. No EC values for Sb in the Sb $_{(Individual)}$ treatment could be calculated due to poor dose-response relationships.

	As $_{(Individual)}$		**As + Sb $_{(Combined)}$**		
	EC_{10} (mg/kg)	**EC_{50} (mg/kg)**	**EC_{10} (mg/kg)**	**EC_{50} (mg/kg)**	**Interaction*, ∑TU $EC_{50(As + Sb)}$**
Tiller number	4 (0-9)	22 (10-33)	9 (0-25)	23 (9-37)	Additive, 1.1 ± 0.3 TU
Root dry mass	0.96 (0-3.63)	8 (0-17)	1 (0-5)	8 (0-18)	Additive, 1.1 ± 0.6 TU
Shoot dry mass	5 (0-11)	25 (14-36)	12 (1-23)	26 (18-35)	Additive, 1.1 ± 0.2 TU
Root length	35 (11-59)	59 (45-72)	40 (35-45)	42 (39-44)	Synergistic, 0.7 ± 0.01TU
Shoot length	39 (23-55)	60 (50-69)	41 (39-42)	42 (41-43)	Synergistic, 0.7 ± 0.004TU
Panicle number	4 (0-8)	17 (9-25)	13 (0-26)	24 (16-31)	Antagonistic, 1.4± 0.2TU
Grain dry mass	5.7 (0.3-11.1)	20 (12-27)	14 (5-24)	25 (19-30)	Antagonistic, 1.2± 0.1TU

* (∑TU $EC_{50\ (As+Sb)}$ = 1 TU) = additive, (∑TU $EC_{50\ (As+Sb)}$ > 1TU) = antagonistic, (∑TU EC_{50} (As+Sb) < 1 TU) = synergistic

6.3.4 Mixture toxicity and interactivity

Toxic units for each endpoint were only calculated for $EC_{50\text{-}As}$ due to the poor concentration-response relationships observed for Sb in the Sb $_{(Individual)}$ treatment. The number of tillers was the same in the concentration-response curve for both the As + Sb $_{(Combined)}$ and the As $_{(Individual)}$ treatments (Figure 6.2b). This suggests that the mixture of As + Sb had an additive toxicity on the number of tillers of *O. sativa.* There was a decrease in tiller number in the Sb $_{(Individual)}$ treatment which was not significant so could not be included in the mixture modelling (Figure 6.3). Therefore, the ∑TU shown in Table 6.3 would be lower if the effect of Sb been included (i.e. the response would have shifted from additivity towards antagonism). For shoot and root dry mass, the EC_{50} of As + Sb $_{(Combined)}$ was equal to 1 TU suggesting an additive interaction (Figure 6.3a and b). However, for shoot and root lengths (Figure 6.3c and d), there was a slight shift in the dose-response curve to the left (EC_{50} of As + Sb $_{(Combined)}$ was less than 1 TU). This suggests that the interactive toxicity of As + Sb was synergistic to *O. sativa* shoot and root lengths. In contrast, a slight shift in the dose-response relationship to the right was observed in the response of *O. sativa*'s number of panicles and grain dry mass (Figure 6.3e and f, respectively). The resulting As + Sb $_{(Combined)}$ EC_{50} of greater than 1 TU indicates that the interactive toxicity of As + Sb is antagonistic.

Although the individual toxicity of As has been well documented, no previous research has investigated the mixture toxicity of As and Sb. Instead a few studies have investigated the mixture metal toxicity of As. For example, the binary mixture of As and Cd was shown to have lower toxicity than their individual exposures (Sun et al. 2008) and synergistic toxicity on wheat root elongation (Cao et al. 2007). Chapter 4 of thesis showed that As and Sb co-contaminated soils had a synergistic effect on *I. aquatica* shoot dry mass under aerobic conditions. Under anaerobic conditions, the predominant As and Sb species are As(III) and Sb(III), respectively. Both of these chemical species are associated with the antioxidant system in cells and form complexes with PCs and sequestration in vacuoles or xylem via silicic acid transporters as discussed in Section1.5.3 (Garg et al. 2011, Zheng et al. 2012, Pigna et al. 2015). Since they use a shared detoxification mechanism, it is possible that presence of both As(III) and Sb(III) in soil had an increased toxicity. However, further investigation on As and Sb toxicity mechanisms in plant cells

are needed. In this study, the waterlogged soils were used to reflect environmental conditions and the redox values were not measured during the experiment. The assumptions were made on the fact that soil is waterlogged and thus, As and Sb are in their reduced state. It is possible that the porewaters were not completely anoxic, and this should be considered when interpreting the results, in the absence of the redox values.

6.3.5 Accumulation of As and Sb in *Oryza sativa* L.

After exposing *O. sativa* to As and Sb for 100 days, both metalloid concentrations in rice grains (includes both husk and the grain) and shoots (plant tissues above the ground excluding panicles) were compared to the SEP-bioavailable concentrations within both the individual and combined treatments.

As per Figure 6.4a, As accumulation in grains ranged from 0.045-1.80 mg/kg and 0.039-1.85 mg/kg in the As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments, respectively. Similarly, these results also showed that As has more affinity to accumulate in rice grains than Sb. Similar to these results, Abedin et al. (2002) reported that As accumulation in rice grains reached 0.42 mg/kg at soil concentrations of 102 mg/kg. In general, Sb accumulation in rice grains increased from 0.02 to 0.96 mg/kg and 0.02 to 0.48 mg/kg in Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments, respectively, with increasing soil Sb concentrations (Figure 6.4b). One sample from the Sb $_{(Individual)}$ treatment had a concentration of 8 mg/kg, which may be due to carryover or contamination of the rice grains and was considered an outlier. A previous study based on As and Sb co-contaminated paddy soils (near a Sb-mining site) showed that more As was accumulated in rice grains than Sb, although this occurred at low total soil As concentrations. For example, average As concentration in rice grains was 0.36 mg/kg at total soil As concentrations of 0.01-57.21 mg/kg and the average Sb concentration in rice grains was 0.05 mg/kg at total soil Sb concentrations of 5.91-322 mg/kg (Wu et al. 2019).

In rice shoots, As concentrations increased with increasing As in the soil in both individual and combined treatments. Shoot As concentrations were similar in both the As $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments at all As exposure concentrations. These results are consistent with those from Abedin et al. (2002) who showed that As accumulation in rice shoots increased from 4-92 mg/kg with increasing soil As from 30-100 mg/kg, respectively.

Similar to As, increased Sb concentrations in soils led to slight increases in rice-shoot Sb concentrations with no difference between individual and combined treatments (excluding two outliers shown in Figure 6.4d). This suggests that As and Sb accumulation is not affected by their co-contamination. While previous studies reported that As and Sb concentrations in rice shoots can reach up to ~4 and ~11 mg/kg, respectively, no information is available comparing their accumulation between individual and co-contamination scenarios (Okkenhaug et al. 2012, Wu et al. 2019).

This research focused on anaerobic conditions and did not compare As and Sb accumulation in rice under both waterlogged and aerobic conditions. However, Xu et al. (2008) has reported that As accumulation in rice shoots and grains were 10-15 folds higher in waterlogged soils. This is likely due to the increased mobility of As under waterlogged conditions. Under aerobic conditions, As(V) can strongly bind on to the Fe(III) (hydr)oxide minerals; however, due to the reductive dissolution of these minerals (reduction of Fe(III) to Fe(II), Figure 1.5), As(V) can be released to the soil solution (Bennett et al. 2012). Therefore, the accumulation results shown in this research are likely to be higher than expected for rice grown under aerobic conditions.

The accumulation of As and Sb in roots was higher compared to shoots and grains (Figure 6.4e). Root As concentrations increased with increasing soil As concentrations until ~30 mg/kg, with not much change at higher exposures. Figure 6.4e and f also showed that there was no difference in the accumulation of either As and Sb in rice roots for $As_{(Individual)}$ and $Sb_{(Individual)}$ exposures, and for $As + Sb_{(Combined)}$ treatments. The high root As concentrations could be from increased accumulation and from soil particles that were not able to be removed from soil particles stuck to root surfaces during the washing step at the end of the bioassay. It should be noted that some of the As may be accumulated in the iron plaque which forms on the surface of the rice roots (Lee et al. 2013). This can sequester As and Sb by adsorption or co-precipitation, often leading to larger concentrations of these metalloids associated with the roots (Liu et al. 2006, Huang et al. 2012). Similar to As, root Sb concentration increased with increasing soil Sb concentrations and no difference was observed in the root Sb content between $Sb_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments (Figure 6.4f). Iron plaque in rice roots could significantly reduce Sb uptake, however, this remains controversial as some evidence suggests Fe plaque formation significantly increase Sb(V) concentration in rice root (Zhu et al. 2020).

Previous studies have shown a similar pattern of As and Sb accumulation, with root> shoot> grains (Abedin et al. 2002, Smith et al. 2008, Okkenhaug et al. 2012, Ren et al. 2014, Cai et al. 2016, Wang et al. 2019, Wu et al. 2019). Rahman et al. (2007) found that root As concentrations were 28 and 75 times higher than that of rice shoots and grains, respectively.

6.3.6 Translocation of As and Sb from *Oryza sativa* L. shoots to grains

Following uptake by roots, As and Sb is transported to shoots and grains via both xylem and phloem transport. The lower concentrations of As and Sb in the grains compared to shoots or roots could be due to a low affinity for inorganic As in the xylem and phloem transport system (Awasthi et al. 2017). The transport of As(III) is known to occur by aquaglyceroporins and further details can be found in Section 0.

The translocation factor (TF) for As and Sb from shoots to grains were measured using the ratio of total concentrations of As/Sb in grains to shoots (Figure A5.3). For As in both the $As_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments, the TF ranged from 0.06 to 0.1 indicating that the co-occurrence of As and Sb had no influence on As translocation from shoots to grain. However, the Sb TF in both $Sb_{(Individual)}$ and $As + Sb_{(Combined)}$ treatments were quite variable with no clear trend with respect to increasing exposure concentration (Figure A5.4). Although total soil Sb concentrations were higher than the total soil As concentrations, relatively lower TFs were observed for Sb.

The lower TF of Sb compared to As obtained at the co-contaminated treatment may be due to the smaller ionic radius of $As(OH)_3$ compared to $Sb(OH)_3$. Although no information is available on the translocation of As and Sb in rice plants comparing individually and co-contaminated soils, Wang et al. (2019) observed a higher TF for Sb from rice shoots to grains and a lower TF for Sb from rice roots to shoots compared to the corresponding TFs for As. This indicates that *O. sativa* roots have a greater transport capacity for As to shoots compared to Sb. In contrast, there was greater Sb translocation from shoots to grains compared to As.

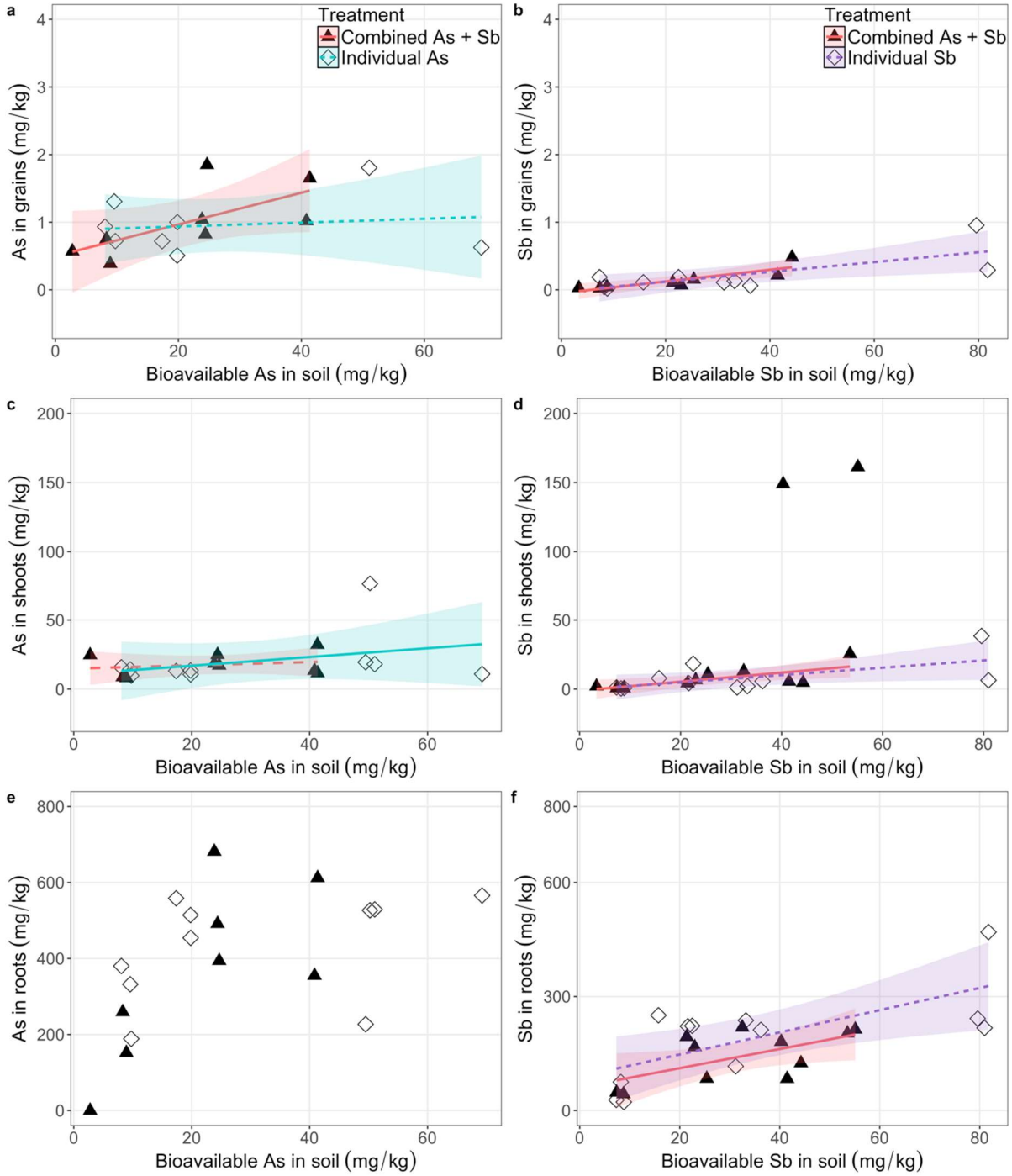


Figure 6.4. The accumulation of a). As and b). Sb in *Oryza sativa* grains; c). As and d). Sb in *O. sativa* shoots; and e). As and f). Sb in *O. sativa* roots after 100 day exposures, compared to SEP-bioavailable concentrations in soils from individual As and Sb and combined As + Sb treatments. A linear regression with 95% confidence intervals is overlaid on these data for their linear range. Outliers were not included in the regression in c) and d) and no concentrations were measured in the highest concentrations of the As + Sb $_{(Combined)}$ treatment due to plant death. Dead plants are not included in this figure. See Table A5.1 and Table A5.2 for all measurements.

6.4 Conclusions

The Chapter 6 of this thesis investigated the interactive effects of As and Sb on the bioavailability, toxicity and accumulation of these metalloids in soil-rice plant systems under waterlogged conditions. The waterlogged soils were used to reflect environmental conditions and the redox values were not measured during the experiment. With hindsight, the redox conditions of the soils should have been measured throughout the experiment, and redox measurements should be included in all future work. As and Sb accumulation in rice roots, shoots and grains under anaerobic conditions was not affected by their co-contamination in soil. Arsenic and Sb accumulation in rice decreased in the order of root > shoot > grain. As and Sb were shown to be toxic to rice decreasing shoot root dry mass and length, tiller and panicle number and grain dry mass; however, the response to Sb was too variable to derive a dose-response relationship. The co-contamination of As and Sb was shown to be acutely toxic at the highest combined concentration. Decreases in all endpoints were also reported at the second highest concentration (C3) in the combined treatment. The co-occurrence of As and Sb had an additive toxicity on tiller number, shoot and root dry mass, synergistic toxicity on shoot and root lengths and antagonistic toxicity on panicle number and grain dry mass. However, these were calculated without the influence of Sb which had highly variable toxicities in individual exposures. Co-contamination however did increase toxicity of the metalloids with plant deaths occurring in soils containing the highest combined concentrations.

Chapter 7. General discussion

There are important knowledge gaps around how environmental and anthropogenic factors can affect the bioavailability of As and Sb in soils and their accumulation and toxicity to agricultural plants (Section 1.6). In this thesis, the bioavailability, accumulation, and toxicity of As and Sb was assessed in soils, both individually and as co-contaminants. A series of studies investigated the bioavailability of As and Sb under various conditions: contaminant ageing in soils, the addition of PO_4^{3-} fertilisers to contaminated soils and soil waterlogging. Agriculturally important plants were used as the test species for these studies including water spinach (*Ipomoea aquatica*), choy sum (*Brassica chinensis var. parachinensis*) and rice (*Oryza sativa*).

7.1 Bioavailability of As and Sb in soil

7.1.1 Use of a sequential extraction procedure (SEP) technique

The bioavailability of As and Sb in soil is partially controlled by their adsorption to soil particles, which is affected by soil characteristics, the chemical speciation and residence time of the metalloids. In natural environments, this adsorption can be altered by environmental conditions including soil ageing, change of redox conditions and the presence of competitive ions. To measure the proportion of total As and Sb concentrations which is bioavailable in contaminated soils, a sequential extraction procedure (SEP) was used throughout this thesis, with the merits and details of this approach discussed in Sections 3.3.2, 4.3.1, 5.3.2 and 6.3.2.

The SEP method of Wenzel et al. (2001) uses a series of solutions with differing extraction strengths and characteristics to extract metals and metalloids (including As and Sb) species bound to various soil-binding fractions (Müller et al. 2007, Wilson et al. 2010, Fu et al. 2016). The metals and metalloids in the non-specifically (SO_4^{2-} extractable) and specifically (PO_4^{3-}extractable) bound fractions usually contain water soluble and ion exchangeable As and Sb species (Wenzel et al. 2001). Therefore, the sum of these fractions are assumed to be representative of the bioavailable fraction of As and Sb in soils (Ngo et al. 2016). In support of this, Tang et al. (2007) also showed that non-specifically and specifically bound As extracted with sequential extraction procedure of Wenzel et al. (2001) had a significant correlation with As bioaccessibility (in both gastric

and small intestinal phases) and are likely to be a good indication of the bioavailable fraction. However, this is only an approximation of the bioavailable fraction which does not necessarily consider factors which may affect bioavailability to specific plants including time frames of exposure and specific plant-soil interactions.

The two main soil types used in this thesis were historically co-contaminated mining soil (aged ~ 34 years) and recently contaminated soils (spiked and aged for 14 days). The recently contaminated soils had three different treatments; As $_{(Individual)}$, Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$. This thesis has shown that the bioavailable fraction of As and Sb in contaminated soil, measured using the SEP method of Wenzel et al. (2001) was able to correlate the biological responses of three agricultural plant species (*I. aquatica* (Section 3.3.3 and 4.3.2), *B. chinensis* (Section 5.3.3), and *O. sativa* (Section 6.3.3)). Further, SEP-bioavailable concentrations were less than total soil concentrations and had a good correlation in recently (aged for ~14 days) contaminated soil used in this thesis. Overall, this confirmed that the extraction method used in this thesis was appropriate for evaluating the bioavailable fraction of As and Sb in the agricultural soils tested here. This work only shows the bioavailable fraction as appropriate for plants.

7.1.2 Impacts of different factors on bioavailability of As and Sb in soil

The measurement of the bioavailable fraction of As and Sb is important to predict plant toxicity, as it is not possible to determine the age of contaminated soils by visual inspection. For both As and Sb, SEP-bioavailable concentrations increased proportionally with the total soil concentrations in all studies of this thesis (Figure 3.1, Figure 4.1,

Figure **5.1** and Figure 6.1). Arsenic and Sb bioavailability in the co-contaminated soil decreased with soil ageing (Section 3.3.2). This is likely due to more mobile As and Sb phases becoming less mobile over time as As and Sb in soil solutions undergo retention mechanisms such as adsorption, precipitation and co-precipitation (Juhasz et al. 2008, Wilson et al. 2010, Wang et al. 2015, Liang et al. 2016). Furthermore, some studies have shown that the formation of inner sphere complexes, precipitation or diffusion into the minerals increases with increased metalloid-soil residence time (ageing), leading to a decrease in the metalloids bioavailability (Juhasz et al. 2008, Naidu et al. 2008, Violante et al. 2008, Wang et al. 2015). Thus, it is clear that although As co-occurs with Sb in

contaminated soil the bioavailability of these metalloids decreases over the time, even at high concentrations.

The Chapter 3 of this thesis investigated soils with total As and Sb concentrations that were higher in historically contaminated soil compared to recently contaminated soils, although the bioavailable fraction was low in both soils. This implies that the bioavailable fractions in soil are better in predicting their potential risk than total soil concentrations.

Simultaneous contamination of As and Sb at elevated concentrations is commonly found in lands impacted by mining activities yet impacts of their interactive effect on bioavailability of As and Sb in soils is unclear in the literature. Previous studies have found that As and Sb have chemical similarities (Telford et al. 2009, Wilson et al. 2010, Doherty et al. 2017). For example, under aerobic conditions, As and Sb have the same chemical speciation (oxidation state of V), but have different affinities for soil sorption sites (Wilson et al. 2010, Kolbe et al. 2011) due to different co-ordination structures of AsO_4^{3-} (tetrahedral) and SbO_4^{3-} (octahedral). The results obtained in Chapter 4 of this thesis showed that there were no competitive interactions observed between As and Sb on their bioavailability. Further, no differences were observed in the bioavailable fractions of As and Sb between individual and co-contaminated soils at the concentrations tested in this research, except for Sb at the highest concentration of the As + Sb $_{(Combined)}$ treatment in the water spinach study (Section 4.3.1). This suggests that, for these test soils, the presence of Sb has no impact on the adsorption of As to soil sorption sites at these concentrations tested while the presence of As decreased the adsorption of Sb only at the highest concentration. Several studies have also reported a decrease in Sb adsorption on to iron oxides in the presence of As (Kolbe et al. 2011, Wu et al. 2018). This could be due to a greater affinity of As for soil sorption sites than Sb possibly due to AsO_4^{3-} being a smaller molecule in the soil solution compared to SbO_4^{3-}.

The addition of PO_4^{3-} via fertilisers alters the soil chemistry, which may impact soils contaminated with As and Sb. This is most significant under aerobic conditions, as AsO_4^{3-} and PO_4^{3-} have similar tetrahedral coordination structures and may compete for soil binding sites (unlike AsO_4^{3-} and SbO_4^{3-}, which have different coordination structures). As a result, PO_4^{3-} may substitute AsO_4^{3-} in adsorption/desorption and precipitation/dissolution reactions as discussed in Section 1.3). Both low and high

concentrations of PO_4^{3-} were investigated here (Chapter 5), with even the high soil PO_4^{3-} concentrations (500 mg P/kg) having little effect on the bioavailability of As and Sb during individual exposures. In co-contaminated soil, PO_4^{3-} decreased As bioavailability at high As exposures whereas Sb bioavailability was not effected by PO_4^{3-} except when Sb in soil ≥1680 mg/kg (Figure 5.1). This contradicts previous findings in the literature which showed that the addition of PO_4^{3-} increased As bioavailability (Rivas-Pérez et al. 2015). In the co-contaminated treatment, only the highest Sb concentration in As + Sb $_{(Combined)}$ treatment (≥1680 mg Sb/kg) was effected by the PO_4^{3-} (Figure 5.1). This trend was also observed in a few studies by showing an increase in the SbO_4^{3-} bioavailability (Kolbe et al. 2011, Arco-Lázaro et al. 2016, Rouwane et al. 2016, Qi et al. 2017) in the presence of both PO_4^{3-} and AsO_4^{3-}.

Soil flooding or waterlogging is common in rice cultivation and this leads to a change in the speciation of As and Sb in soils, due to the lack of oxygen. As and Sb co-contamination had little to no influence on their bioavailable fractions compared to the individual treatments under waterlogged conditions. Competitive interactions were expected in Chapter 6 of this thesis due to the similar chemical speciation (+III) and coordination structures of As and Sb under anaerobic conditions (as discussed in Section 1.2.4). Under waterlogged (anaerobic) conditions, the reductive dissolution of iron oxides (i.e. reduction of Fe(III) to Fe(II)) of the soil minerals release As from Fe-As complexes, thereby increasing their bioavailability (Section 0) (Xu et al. 2008). Although increased bioavailability was expected, in this thesis the bioavailability of As and Sb measured under aerobic conditions (Chapter 4) was not different to those reported under anaerobic conditions (Chapter 6), see Figure 4.1 compared to Figure 6.1.

This thesis has shown As and Sb bioavailability decreased with soil ageing and addition of high PO_4^{3-} concentrations (only at high soil As and Sb concentrations) whereas no significant difference was observed within co-contaminated soils compared to their individually contaminated soil at both aerobic and waterlogged conditions.

7.1.3 Accumulation and toxicity of As and Sb to different agricultural plants

In this thesis, differences in As and Sb accumulation and toxicity under various environmental conditions was assessed using three plant species, *I. aquatica* (water

spinach, Chapters 3 and 4), *B. chinensis* var. *parachinensis* (choy sum, Chapter 5) *and O. sativa* (rice, Chapter 6). A summary of the accumulation concentrations in these plants under different conditions are shown in Table 7.1 and summary of effective concentrations which caused a 50% decrease in growth endpoints (EC_{50}) also provided in Table 7.2. However, care must be taken when considering these results as this thesis has used oven drying at 60 C to dry plant samples. The oven drying method may increase losses of volatile species of As and Sb in the plant and hence data should be interpreted accordingly. Future research should use freeze drying as the preferred approach for tissue preparation if accessible.

7.1.4 Impacts of different factors on the accumulation

Arsenic and Sb are known as non-essential elements; however, they can be readily accumulated in the edible parts of agricultural plants posing a risk to consumers. A higher bioavailability of metalloids in soil usually represents a higher fraction of water soluble As and Sb species which is readily available for plant uptake (Section 1.4.1). In historically co-contaminated soil, *I. aquatica* shoots (edible part) accumulated 163 and 27 mg/kg of As and Sb while in recently co-contaminated soil they accumulated 78 and 100 mg/kg of As and Sb, respectively. The accumulation of As by *I. aquatica* in historically co-contaminated soils increased with the exposure concentration, while that of recently contaminated soils showed a decrease at higher exposure concentrations due to a decrease in plant biomass (toxicity). There is a greater accumulation and risk of dietary exposure to humans from recently contaminated compared to historically contaminated soils of equivalent concentrations because of the decreased As and Sb bioavailability with soil ageing (increasing metalloid residence time) in co-contaminated soil (Section 3.3.2).

Arsenic accumulation in *I. aquatica* (after 35 d exposure) was not affected by As and Sb co-contamination while Sb accumulation increased in co-contaminated soils at high concentrations compared to individual Sb exposures (Chapter 4). This may be because of the preferential binding of As over Sb to soil binding sites leading to an increase of Sb uptake in the presence of As (see Figure 4.1). However, care must be taken when considering these results as the highest bioavailable Sb concentration in the As + Sb $_{(Combined)}$ soil was higher than Sb $_{(Individual)}$ contaminated soil (Table 4.1). Similar to the

results obtained in Section 4.3.5, previous studies observed an increase of Sb uptake by in hyperaccumulating ferns, *P. cretica* and *P. vittata* grown in As and Sb co-contaminated soil (Feng et al. 2011, Muller et al. 2013).

In individually contaminated soil, the accumulation of As and Sb increased at high PO_4^{3-}, whereas in co-contaminated soil, As and Sb accumulation increased only within the low soil concentrations (total soil: ≤ 240 mg As/kg and ≤ 244 mg Sb/kg) (Chapter 5). The bioaccumulation factor (BAF) for As increased with the addition of PO_4^{3-} for most of the concentrations in both individual and combined treatments. This suggests that As transport from soil to roots was greater with a higher PO_4^{3-} concentration in the soil. This is consistent with similar studies also showing that the addition of PO_4^{3-} to soil contaminated with As increased As accumulation in plants including wheat root and shoots and chickpea shoots (Cao et al. 2004, Tao et al. 2006, Gunes et al. 2009, Pigna et al. 2009). This was mainly due to an increase in bioavailable As from the replacement of AsO_4^{3-} by PO_4^{3-} on soil binding sites, which was not explicitly measured in this thesis. Increased soil PO_4^{3-} led to a lower translocation of As from *B. chinensis* roots to shoots which may be due to higher affinity PHT transporters in *B. chinensis* for PO_4^{3-} than AsO_4^{3-}. Further, the reduction of AsO_4^{3-} (As(V)) to AsO_3^{3-} (As(III)) and subsequent vascular sequestration of As(III)–PC complex in the roots (Garg et al. 2011, Zheng et al. 2012, Pigna et al. 2015) could also reduce the translocation of As from roots to shoots.

A greater BAF was observed for Sb in the individually contaminated soil whereas the BAF decreased for Sb within co-contaminated soils at high PO_4^{3-} concentrations. However, there is no evidence to suggest that PO_4^{3-} and $Sb(OH)_6^-$ uptake occurs through the same transporters in the roots due to their different chemical structure and size (Tschan et al. 2008, Tisarum et al. 2015). In support of this, Feng et al. (2013) reported that $Sb(OH)_6^-$ uptake did not appear to occur via phosphate pathways and Tschan et al. (2008) showed that the addition of PO_4^{3-} did not affect the uptake of Sb in maize and sunflower plants.

Under waterlogged conditions, the accumulation of As and Sb in *O. sativa* (after 100-days exposure) was not affected by soil co-contamination (Chapter 6). Under anaerobic conditions, the oxyanions of both As and Sb have similar coordination structures and thus, competition between them for uptake is likely. It is theorised that As may compete with

Sb for uptake through aquaglyceroporins (as described in Section 1.4.2), altering As accumulation when present in co-contaminated soils (Feng et al. 2011). However, this was not seen in this thesis.

Arsenic and Sb accumulation in plants is conventionally understood to increase under waterlogged anaerobic conditions. For example, Xu et al. (2008) reported As accumulation in rice shoots and grains were 10-15 folds higher in waterlogged soils compared to aerobic soils. This may be due to the increased mobility of As under waterlogged conditions. However, this thesis did not directly compare As and Sb accumulation in *I. aquatica* and *O. sativa* under both aerobic and anaerobic conditions in the same experiment set up and thus, this warrants further investigation. The different amounts of As and Sb taken up by the different plant species are shown in Table 7.1. This suggests that uptake of As and Sb in plants are dependent on plant and metalloid species.

In conclusion, As and Sb concentrations in the edible parts of tested agricultural plants decreased with soil ageing whereas with recently contaminated soils there was an increase. However, no change in As and Sb accumulation were observed in co-contamination scenarios and waterlogged condition. Thus, consumption of agricultural plant species such as *I. aquatica* grown in aged soil poses a lower risk compared to plants grown in recently contaminated soils under variety of different environmental conditions.

7.1.5 Impacts of different factors on the toxicity

The toxic effects of As to plants have been reported as inhibited root extension, reduced fertility, yield, and fruit production, and overall reductions in plant growth and biomass (Finnegan et al. 2012). The toxic effects of Sb have been mainly associated with reduced growth and reduced synthesis of some metabolites (Feng et al. 2013). These toxic effects vary between plant species and are dependent on environmental conditions and the bioavailability of the metalloids within the soil. In this thesis, the toxicity of As and Sb to plants in laboratory studies were determined quantitatively by investigating changes in tissue dry mass, shoot and root length and other physiological measurements, including chlorophyll content and photosynthetic efficiency. Arsenic was toxic at the exposure concentrations tested in this thesis, with clear dose response relationships derived for all three plant species (*I. aquatica*, *B. chinensis* and *O. sativa*). Antimony was only shown to be toxic to *O. sativa*, although a clear dose-response relationship was not derived due

to high variability of the response. This shows that As is generally more toxic to agricultural plants than Sb, which agrees with trends in the literature (He 2007, Feng et al. 2013).

Different plants have different sensitivities to As. Inhibition of shoot dry mass was a common toxicity endpoint for the three plant species tested in this thesis; *I. aquatica, B. chinensis* and *O. sativa* with estimated EC_{50} values of 81(56-106), 42 (21-63) and 25 (14-36) mg As/kg, being reported respectively (Table 7.2). These results suggest that *O. sativa* was the most sensitive plant species to As of those tested in this thesis. Although other endpoints also showed toxicity, some of their concentration-response relationships were too variable to derive EC values (Figure A4.3, Figure A5.2 and Figure A5.3).

Soil ageing and the addition of PO_4^{3-} decreased toxicity of As to *I. aquatica* and *B. chinensis,* respectively. Decreased toxicity of As and Sb to *I. aquatica* was observed in historically contaminated compared to recently contaminated soil (Chapter 3). For example, the inhibition of *I. aquatica* shoot dry mass EC_{50} for historically (aged for ~34 years) and recently (recently contaminated and aged for 14 days) co-contaminated soil were observed at 49 and 20 mg As/kg, respectively (Table 7.2).

At high soil PO_4^{3-} concentrations across the concentration gradient, As was not toxic to shoot and root dry mass (Chapter 5). These results were supported by Gunes et al. (2009) who reported that PO_4^{3-} may have partially protected chickpea plant growth from inhibition by As, by reducing oxidative stress to cellular membranes. Furthermore, a couple of studies have shown that soybean and sunflower plant biomass increased with PO_4^{3-} addition to As contaminated soils (Azeem et al. 2017, Kamran et al. 2018). However, opposite results were observed by Cao et al. (2004) who showed that the addition of PO_4^{3-} (at high concentration, 3867 mg/kg) induced As toxicity to carrot and lettuce plant biomass due to increase As accumulation in plant tissues. This suggests that the addition of high PO_4^{3-} to contaminated soils can ameliorate As toxicity to certain plants such as *B. chinensis* used in Chapter 5, however, this is not the case for all plant species.

Table 7.1. Accumulation of As and Sb in different plant species. Length of exposure for *Ipomoea aquatica*- 35 days, *Brassica chinensis* var. *parachinensis*- 40 days and *Oryza sativa*- 100 days. Total daily intake values for As and Sb calculated based on 46 kg body weight (Wu et al. 2011, Ngo 2018). The units of As and Sb are presented in mg/kg. Values that exceed the recommended TDI (129 ug As/day and 360 ug Sb/day) are in bold.

	As (Individual)			**As + Sb (Combined)**			**Sb (Individual)**			**As + Sb (Combined)**		
	Soil As (mg/kg)	**As in edible part (mg/kg)**	**Total daily intake (µg/kg)**	**Soil As (mg/kg)**	**As in edible part (mg/kg)**	**Total daily intake (µg/kg)**	**Soil Sb (mg/kg)**	**Sb in edible part (mg/kg)**	**Total daily intake (µg/kg)**	**Soil Sb (mg/kg)**	**Sb in edible part (mg/kg)**	**Total daily intake (µg/kg)**
I. aquatica	[a]	[a]	[a]	42.2-2630	4-160	130-5335	[a]	[a]	[a]	40-2920	0.7-27	23-900
I. aquatica	45-420	1-80	470-2700	60-405	17-80	570-2665	30-1200	0.8-14	30-465	35-1250	0.6-100	20-3333
B. chinensis	55-335	2-47[c] 2.3-31[d]	70-1570[c] 80-1030[d]	53-330	5-25[c] 3-23[d]	170-835[c] 100-770[d]	80-2220	5-13[c] 0.46-2[d]	170-435[c] 15-65[d]	163-1680	1.3-15[c] 0.55-24[d]	44-500[c] 18-800[d]
O. sativa	31-130	0.05-1.80	[b]	32-116	0.04-1.85	[b]	30-71	0.02-0.96	[b]	30-55	0.02-0.48	[b]

[a] Not available, as aged soil bioassays were established with As and Sb co-contaminated soil, [b] Cannot calculate as the grains contained husk , [c] *B. chinensis* at high soil PO_4^{3-} concentrations, [d] *B. chinensis* at low soil PO_4^{3-} concentrations

Table 7.2. Toxicity of As in individual (As $_{(Individual)}$) and combined (As + Sb $_{(Combined)}$) exposures to plant species with bioavailable concentrations that cause a 10% and 50% (EC_{10} and EC_{50}, with 95% confidence intervals (CI)) reduction to endpoints. NA- not available as historically contaminated soils were obtained from abounded mining area where both As and Sb were present.

Plant species	**End points**	**As $_{(Individual)}$**		**As + Sb $_{(Combined)}$**	
		EC_{10} (mg/kg)	**EC_{50} (mg/kg)**	**EC_{10} (mg/kg)**	**EC_{50} (mg/kg)**
***Ipomoea aquatica* (historically contaminated)**	Shoot dry mass	NA	NA	4 (1-12)	49 (37-65)
	Shoot length	NA	NA	4 (0.6-31)	96 (64-145)
	Root dry mass	NA	NA	9 (2-46)	83 (53-120)
	Root length	NA	NA	5 (8 -121)	133 (91-194)
***Ipomoea aquatica* (recently contaminated)**	Shoot dry mass	10 (0-22)	81 (56-106)	0.4 (0-1.2)	20 (7.4-33)
	Shoot length	2 (0-6)	65 (38-94)	3 (0-7)	72 (43-101)
Brassica chinensis* var. *parachinensis	Shoot dry mass	19 (2-36)	42 (21-63)	49 (15-85)	72 (53-91)
	Root dry mass	20 (0-40)	40 (17-62)	20 (0-47)	56 (24-88)
Oryza sativa L.	Tiller number	4 (0-9)	22 (10-33)	9 (0-25)	23 (9-37)
	Root dry mass	0.96 (0-3.63)	8 (0-17)	1 (0-5)	8 (0-18)
	Shoot dry mass	5 (0-11)	25 (14-36)	12 (1-23)	26 (18-35)
	Root length	35 (11-59)	59 (45-72)	40 (35-45)	42 (39-44)
	Shoot length	39 (23-55)	60 (50-69)	41 (39-42)	42 (41-43)
	Panicle number	4 (0-8)	17 (9-25)	13 (0-26)	24 (16-31)
	Grain dry mass	5.7 (0.3-11.1)	20 (12-27)	14 (5-24)	25 (19-30)

Overall, this thesis has demonstrated that As is more toxic than Sb to all three plant species. Among all toxicity endpoints, shoot dry mass was the most comparable because it followed a good concentration-response relationship for all three of the plant species tested and thus is a good comparative indicator of As toxicity.

7.2 Interactive effect of As and Sb in co-contaminated soil on agricultural plants

The presence of contaminant mixtures can alter the expected toxicity compared to their individual exposures and does not always reflect the expected toxicities of the individual components. As a result, the toxicity of metalloid mixtures may be equal to the sum of the toxic effects of individual components or higher/lower than the sum due to synergistic or antagonistic interactions, respectively. A few studies have investigated the metal mixture toxicity of As. For example, the binary mixture of As and Cd was shown to have lower toxicity than their individual exposures (Sun et al. 2008) and synergistic toxicity on wheat root elongation (Cao et al. 2007). Exposure to Sb individually had no observed toxicity in *I. aquatica* and *B. chinensis* while a poor concentration-response relationship was observed for *O. sativa* (Figure A5.2 and Figure A5.3). Thus, Sb toxicity was not incorporated in mixture toxicity calculations assuming Sb does not have toxicity on *O. sativa* (due to poor concentration-response relationship). However, it should be noted that if the toxicity of Sb was established and the value used in the calculations, it could change the TU.

For shoot dry mass, As in co-contaminated soil had a greater toxicity to *I. aquatica* (EC_{50}: 20 (7.4-33)) compared to its individual exposure (EC_{50}: 81 (56-106)) (Table 7.2, Section 3.3.5). In contrast, As in co-contaminated soil was less toxic to *B. chinensis* compared to its individual exposures (Table 7.2, Section 5.3.3). There was no significant difference in toxicity shown for *O. sativa* between individually and co-contaminated soils (Table 7.2, Section 6.3.3). These results suggest that the effects of As on shoot dry mass in co-contaminated soils are plant specific. For shoot length, As in co-contaminated soil had a lower toxicity to *I. aquatica* (EC_{50}: 72 (43-101)) compared to its individual exposure (EC_{50}: 65 (38-94)) whereas a greater toxicity was observed for *O. sativa* in co-contaminated (EC_{50}: 42 (41-43)) compared to their individual exposures (EC_{50}: 60 (50-69)). EC values for *B. chinensis* were not calculated due to poor concentration-response relationships. Opposite observations for shoot dry mass and length between the plants *I. aquatica a*nd *O. sativa* may be due to different toxic mechanisms in plant species, and this warrants further investigation. These toxic mechanisms may be associated with

mineral nutrition, inhibition of enzyme activity and reduction of photosynthesis activity (Chibuike et al. 2014), although no significant difference was shown in this thesis (Section 3.3.4 and 4.3.4). In addition, different trends (EC_{50} values) were also observed for other endpoints including root dry mass and length (Table 7.2). Based on the range of EC_{50} values for different toxicity endpoints, shoot dry mass was the most sensitive endpoint for *I. aquatica* while root dry mass for *O. sativa* and *B. chinensis* (Table 7.2). This suggests that care must be taken when choosing toxicity endpoints to use in bioassays as the most sensitive endpoint is dependent on the plant species.

When considering the interactive effect of As and Sb in the mixture on *I. aquatica* and *O. sativa* at aerobic and waterlogged conditions, both plant species showed opposite interactions. For example, As and Sb co-contamination in soils was shown to have a synergistic effect on shoot dry mass in *I. aquatica* (Chapter 4) but had an additive interactive effect in *O. sativa* (Chapter 6). Considering shoot lengths, the opposite effect was observed with an additive interaction shown for *I. aquatica* while synergistic interactions were shown for *O. sativa.* The contrasting interactive effects of As and Sb on shoot dry mass and length for *I. aquatica* and *O. sativa* may be due to the different redox conditions at the soil root interface or different plant species biochemistries. However, there is lack of information available on the mixture toxicity of Sb in metal mixtures, and further studies are necessary.

Under aerobic conditions, both As and Sb are predominantly present as pentavalent oxyanions. Both species (As(V) and Sb(V)) can disturb the uptake of essential nutrient in the plant. This may be why As and Sb in co-contaminated soil had a synergistic toxicity in *I. aquatica* shoot dry mass and additive toxicity to shoot length. Since the interactions of As and Sb may occur at different points during uptake to the target cell sites (Cao et al. 2007), they may have contrasting effects on different endpoints. Under anaerobic conditions, the predominant As and Sb species are As(III) and Sb(III), respectively. Both of these chemical species cause toxicity to the antioxidant system in cells, form complexes with PCs and are sequestered in vacuoles or the xylem via silicic acid transporters (Garg et al. 2011, Zheng et al. 2012, Pigna et al. 2015). Since they use a shared detoxification mechanism, it is possible that presence of both As(III) and Sb(III) in soil had competitive interactions. Further investigation on As and Sb toxicity mechanisms in plant cells are needed.

Overall, the effects of As in the As and Sb co-contaminated soil was shown to be plant specific and their interactive effect could be additive or synergistic effect, with the two plant species used in this thesis having different responses.

7.3 Risks of As and Sb to food safety and human health

The values of total daily intake of As and Sb in individually and co-contaminated soils from *I. aquatica* and *B. chinensis* were calculated as per Wu et al. (2011) and are shown in Table 7.1. The total daily intake values were calculated assuming, 30 g of *I. aquatica* and *B. chinensis* is ingested daily based on a person with a body weight of 46 kg (Marcussen et al. 2008). The total daily intake o values of As for *I aquatica* grown in both histrorically and recently co-contaminated soils exceeded the recommended tolerable daily intake (TDI) of As, 129 µg As/day by the Joint Food and Agricultural Organization/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) (Wu et al. 2011). This indicates that although soil ageing reduced the bioavailability of As, cultivation of *I. aquatica* in historically co-contaminated soils still poses a risk to humans via consumption. Increased consumption of As in human body can cause skin lesions, cancers in lungs and bladders and chromosomal changes (Samal et al. 2011).

The total daily intake values of Sb in water spinach grown in historically and recently contaminated soils ranged between 23-900 and 20-3330 µg/day, respectively. These results showed that *I. aquatica* grown in contaminated soils containing low Sb concentrations (≤1400 mg/kg) were well below the TDI (360 µg Sb/day, (Wu et al. 2011)). Plants grown in soils with Sb concentrations > 1400 mg/kg contained Sb concentrations above the TDI. This indicates that the concentrations of As and Sb accumulated in the edible parts of *I. aquatica* are still very high regardless of the soil age for concentrations used in Chapter 3. Although, historically contaminated soils (~34 years post mine usage) decreased As and Sb bioavailability which led to decreased accumulation and toxicity (Figure 3.1).

The total daily intake values for As in *I. aquatica* were not significantly different between individually and co-contaminated soils, but there was a greater risk from Sb in the co-contaminated soil compared to individually contaminated soils, which had a total daily intake value of 3333 µg Sb/day (Chapter 4 and Table 7.1). This risk could be exacerbated

because there were no signs of toxicity in the *I. aquatica* grown even at the highest Sb concentration. These results suggest that *I. aquatica* can tolerate relatively high tissue concentrations of Sb in its edible parts, without any visible toxic deformities. Thus, *I. aquatica* cultivation in Sb-contaminated soil should be avoided or regular testing implemented to prevent dietary exposures.

The addition of PO_4^{3-} had little effect on the bioavailability of As but increased its accumulation in *B. chinensis*. The increased PO_4^{3-} fertiliser concentration used in this thesis also ameliorated the toxicity of As to the growth endpoints shoot root dry mass and length and improved the physical appearance of *B. chinensis*. Further, the high total daily intake values for As (Table 7.1) indicate that even at high PO_4^{3-} concentration, the consumption of *B. chinensis* exceeds the TDI posing a risk to human health by accumulating As at high concentrations. This is important to consider as the plants appear healthy yet accumulate high concentrations of As and Sb in their edible tissues. This may be prevented by limiting the addition of PO_4^{3-} to As contaminated soils; however, this requires further research to confirm appropriate PO_4^{3-} dose thresholds. It was not appropriate to calculate the total daily intake values for *O. sativa* as the rice metalloid concentrations were calculated based on the husk as well as the grains.

This thesis suggests that highly contaminated As and Sb soil poses a risk to human health from the consumption of *I. aquatica* and *B.chineses*. Furthermore, this thesis showed that As and Sb have synergistic and additive interaction effects on plant growth but interaction effects of As and Sb on the human body is still unknown.

7.4 Future directions

This thesis investigated the bioavailability, accumulation and toxicity of As and Sb individually and combined to three plant species; *I. aquatica* (water spinach, Chapter 3 and 4), *B. chinensis* (choy sum, Chapter 5) and *O. sativa* (rice, Chapter 6) under various environmental conditions including ageing (historical and recent contamination), the addition of PO_4^{3-}, and waterlogging (anaerobic soils). However, other environmental and farming practices may also affect the risk of As and Sb in agricultural settings. Future research should focus on investigating the following:

- Organic matter can change the physicochemical environment by changing soil acidity and can act as an organic ligand for binding of metalloids within the soil compartment Section 1.3.2. Therefore, future research should explore the effect addition of organic matter such as compost to contaminated soils has on the bioavailability, accumulation and toxicity of As and Sb to agricultural crops.
- Crop rotation can affect the chemical composition of soil as different plant species have different affinities for contaminants such as metals (Zang et al. 2015). These changes may affect As and Sb concentrations and bioavailability within agricultural soils and thus should be investigated further.
- Sb concentrations at Sb mining sites co-contaminated with As can reach concentrations higher than investigated in this thesis. Further studies should be conducted using a greater range of concentrations and different As:Sb ratios.
- Increased Sb accumulation in *I. aquatica* shoots and roots was found in response to application of 500 mg P/kg of PO_4^{3-} to the soil. However, no decrease in growth, as indicated by shoot and root dry mass and length, was observed. The mechanisms behind the enhanced accumulation of Sb in the presence of high PO_4^{3-} is currently unknown and therefore more research into the mechanisms of uptake and translocation for understudied contaminants like Sb is necessary especially under different environmental conditions and farming practices.
- A greater understanding of the metabolism and detoxification mechanisms of Sb in plants are needed especially when grown in soils co-contaminated with As. This is important to understand the role of Sb on As toxicity during co-exposures due to the synergistic and additive interactivity observed in this thesis.
- Arsenic and Sb in individual and combined exposures was shown to have no toxic

effect on the photosynthetic efficiency of plant tested in this thesis. This contradicts previous reports (Pan et al. 2011, Feng et al. 2013) which have suggested that Sb can affect photosynthesis in plants by inhibiting chlorophyll synthesis and maximum photochemical efficiency (expressed as Fv/Fm, Chapter 3.3.4). This suggests that the effect of Sb to photosynthesis may be plant specific and may depend on the detoxifying mechanisms of each plant. Therefore, studies investigating plant specific effects and detoxifying mechanisms of Sb in *I. aquatica* leaves should be conducted to improve understanding of Sb effects on photosynthesis.

- This thesis has investigated the accumulation and toxicity of As and Sb to leafy vegetables *I. aquatica* and *B. chinensis* and the grain *O. sativa*. Root vegetables may also be easily exposed to As and Sb in contaminated soils and these metals may accumulate in and on their edible parts. Limited studies have been published on this topic (Ngo et al. 2016). Therefore, it is important to look at the effects of As and Sb on root vegetables to assess and avoid the potential risks of these metalloids to human and ecosystem health.
- This thesis showed that As and Sb accumulation in rice plant was not affected by co-contamination, nor bioavailability. Further, both metalloids were toxic to rice plant while co-contamination increased toxicity. Therefore, future work should also employ pore water samplers which can be used to explore linkages between water chemistry in waterlogged experiments such as those outlined in Chapter 6. In my most recent research, I applied in situ passive sampling technique of diffusive gradients in thin films (DGT), to assess the labile concentrations and distribution of elements in waterlogged soils. In DGT, the labile fraction of elements passes through a membrane filter and accumulate in the binding gels and this technique is also known as a well suited in situ detection of bioavailable elements. For this study pore water samplers can also be used to explore linkages between water chemistry in the waterlogged experiment (in Chapter 6) which was difficult to explain with sequential extraction.
- This thesis investigated SEP- extractable As and Sb concentrations at the start of the bioassay. However, it would be worthy to assess changes in soils after waterlogging to see the impact of waterlogging on As and Sb binding with different fractions.

7.5 Conclusion

This thesis aimed to investigate the influence of environmental and anthropogenic factors including soil ageing, co-contamination, PO_4^{3-} addition and waterlogging to understand their impacts on As and Sb bioavailability in soil and thereby, on their accumulation and toxicity to agricultural plants. Soil ageing was shown to decrease As and Sb bioavailability, accumulation and toxicity in *I. aquatica,* whereas increased PO_4^{3-} concentrations in As and Sb individually contaminated soil had no impact on As and Sb bioavailability in soil. Increased PO_4^{3-} concentrations in As and Sb co-contaminated soil did decrease As and Sb bioavailability when exposed to high concentrations of As and Sb while increasing their accumulation in shoots posing a greater risk to consumers. Co-contamination of As and Sb had no impact on bioavailability in soil and accumulation in edible parts of agricultural plants used in this thesis. In general, As accumulation and toxicity to all tested agricultural plants grown under different soil conditions was much higher than that of Sb.

O. sativa was the most sensitive plant species during As-individual exposures. Antimony was not toxic to *I. aquatica* and *B. chinensis*, but toxic effects were observed on shoot and root dry mass of *O. sativa.* PO_4^{3-} increased As and Sb accumulation, however, no toxic effects were visually observed increasing the risk As and Sb may pose to human health through dietary exposure.

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Appendix

Appendix 1. Supplementary information for Chapter 2

Table A1.1. Suppliers of the chemicals used in the experiment. All chemicals were analytical reagent grade or higher.

Chemical	Supplier
sodium arsenate	Thermo fisher
potassium hexahydroxoantimonate	Sigma-Aldrich
$(CO(NH_2)_2$	Sigma-Aldrich
$NH_4H_2PO_4$	Ajax FineChem
K_2SO_4	Ajax FineChem
$(NH_4)_2SO_4$	Thermo fisher
NH_4-oxalate	Ajax FineChem
ascorbic acid	Ajax FineChem

Appendix 2. Supplementary information for Chapter 3

Table A2.1. Nominal and final measured concentrations obtained by total soil acid digestion in historically and recently contaminated bioassay soils (mean ± SD, n ≥ 3).

	Nominal concentration (mg/kg)		Total soil concentration (mg/kg)		Accumulated in shoots mg/kg (dry mass)	
	As	**Sb**	**As**	**Sb**	**As**	**Sb**
Historically contaminated soils (mg/kg)						
Control		[a]	5.4 ± 0.6	0.23 ± 0.02	0.5 ± 0.2	0.19 ± 0.05
HS1		50	42.2 ± 0.3	40 ± 8	3.8 ± 0.7	0.66 ± 0.05
HS2		100	71 ± 4	70 ± 1	4.4 ± 0.3	0.4 ± 0.2
HS3		150	100 ± 15	100 ± 10	6 ± 1	0.29 ± 0.03
HS4		200	160 ± 50	160 ± 30	12 ± 1	0.45 ± 0.2
HS5		250	235 ± 60	150 ± 50	15 ± 3	0.54 ± 0.08
HS6		300	270 ± 15	210 ± 80	42 ± 7	2 ± 1
HS7		400	315 ± 35	410 ± 240	36 ±10	1.5 ± 0.4
HS8		500	520 ± 50	510 ± 100	47 ± 4	1.15 ± 0.06
HS9		600	670 ± 220	670 ± 230	50 ±7	1.32 ± 0.07
HS10		800	950 ± 90	900 ± 80	73 ±7	2.3 ± 0.3
HS11		1200	1280 ± 70	1230 ± 80	90 ± 10	1.9 ± 0.5
HS12		1600	1410 ± 90	1750 ± 170	126 ±6	10 ± 4
HS13		2000	2630 ± 150	2920 ± 240	160 ± 23	27 ± 9
HS14		3500	4200 ± 200	5090 ± 500	726	348
Recently contaminated soils (mg/kg)						
Control	[a]	[a]	6 ± 0.3	0.3 ± 0.03	0.42 ± 0.07	0.7 ± 0.1
RS1	40	40	60 ± 10	35 ± 5	16 ± 2	0.62 ± 0.09
RS2	80	80	88 ± 3	56± 10	37± 24	1.0 ± 0.5
RS3	160	160	175 ± 2	130 ± 7	65 ± 10	1.9 ± 0.6
RS4	200	300	220 ± 7	240 ± 5	80 ± 7	4.4 ± 1.5
RS5	300	600	315 ± 5	250 ± 10	70 ± 15	8.9 ± 0.6
RS6	400	1500	405 ± 4	1250 ± 30	40 ± 7	100 ± 18

[a] Control soils.

Table A2.2.

(A). Percentage hydration of water spinach roots and shoots in historically contaminated soils.

Sample	Root			Shoot		
	Wet mass	Dry mass	% moisture	Wet mass	Dry mass	% moisture
Control	4.33	0.78	82.09	47.19	4.21	91.08
HS1	6.20	0.60	90.26	61.42	4.38	92.86
HS2	5.12	0.73	85.74	34.34	3.34	90.26
HS3	8.13	0.90	88.99	45.10	3.93	91.28
HS4	5.58	0.55	90.18	38.13	3.05	91.99
HS5	8.69	0.62	92.85	37.22	2.97	92.01
HS6	9.73	0.91	90.66	49.43	4.54	90.82
HS7	7.97	0.57	92.84	40.23	2.77	93.12
HS8	7.90	0.69	91.31	41.15	2.94	92.86
HS9	6.17	0.58	90.65	28.38	1.99	93.00
HS10	4.53	0.40	91.15	19.66	1.27	93.53
HS11	6.57	0.56	91.49	19.68	1.45	92.64
HS12	4.07	0.28	93.08	19.31	1.41	92.72
HS13	0.94	0.06	93.68	3.97	0.40	89.86
HS14	0.51	0.02	96.66	1.11	0.08	92.71

(B) Percentage hydration of water spinach roots and shoots in recently contaminated soils

Sample	Root			Shoot		
	Wet mass	Dry mass	% moisture	Wet mass	Dry mass	% moisture
Control	4.13	1.46	64.54	45.10	5.84	87.04
RS1	1.49	0.88	40.93	39.17	2.72	93.05
RS2	2.92	0.89	69.52	34.98	2.79	92.08
RS3	3.89	1.07	72.49	30.23	2.75	90.93
RS4	4.15	1.28	69.15	28.34	2.26	92.00
RS5	3.11	1.00	67.84	16.08	1.62	89.93
RS6	0.26	-[a]	-[a]	2.80	0.85	69.64[a]

[a] Measurement error

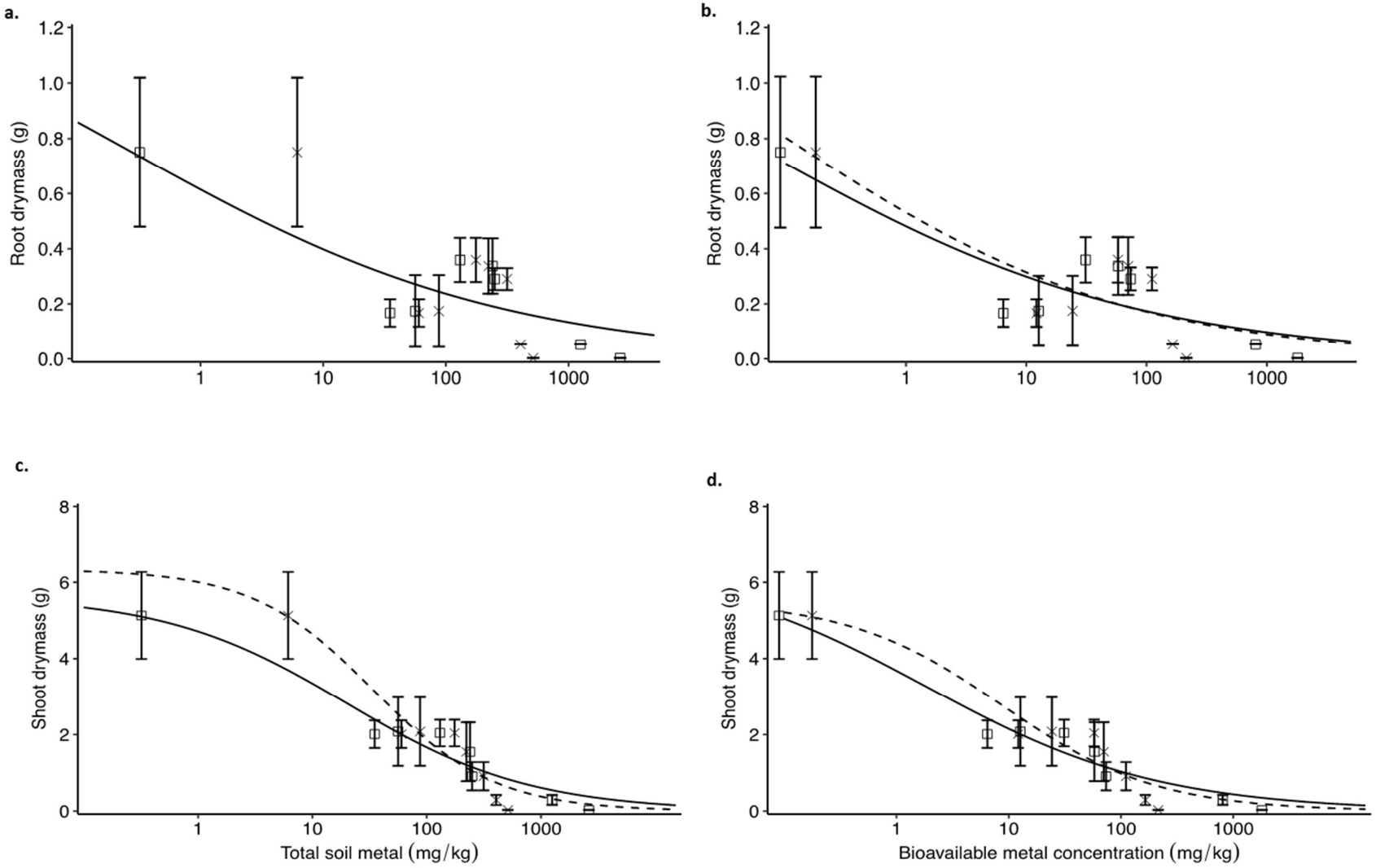

Figure A2.1. The relationship of *Ipomoea aquatica* root and shoot dry mass with (a, c) total and (b, d) SEP-bioavailable concentrations in recently co-contaminated bioassay soils. As (×, dashed line) and Sb (□, solid line). Dry mass reported as mean ± SD, n ≤ 3.

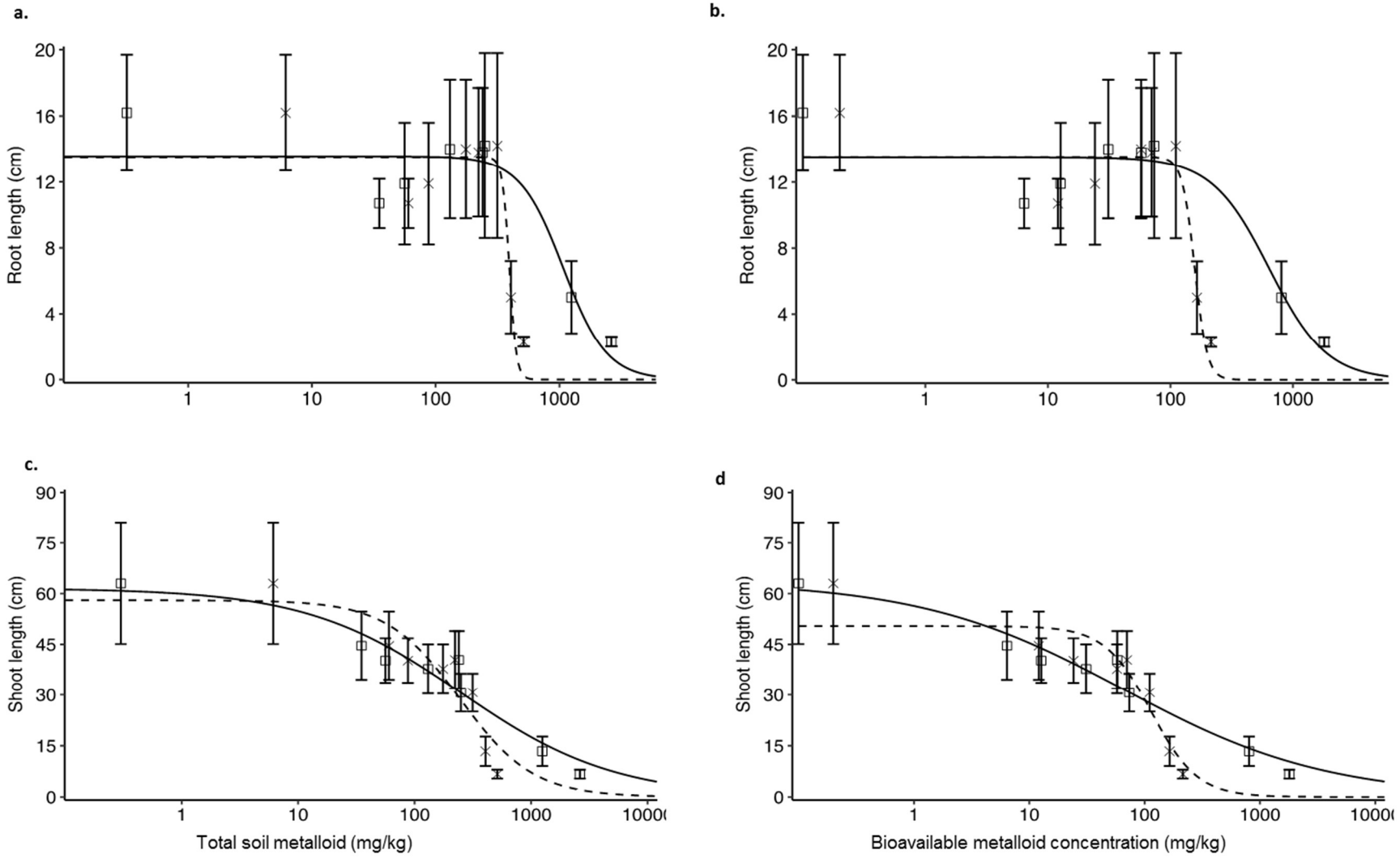

Figure A2.2. The relationship of *Ipomoea aquatica* root and shoot length with (a, c) total and (b, d) SEP-bioavailable concentrations in recently contaminated bioassay soils. As (×, dashed line) and Sb (□, solid line). Tissue length reported as mean ± SD, n ≤ 3.

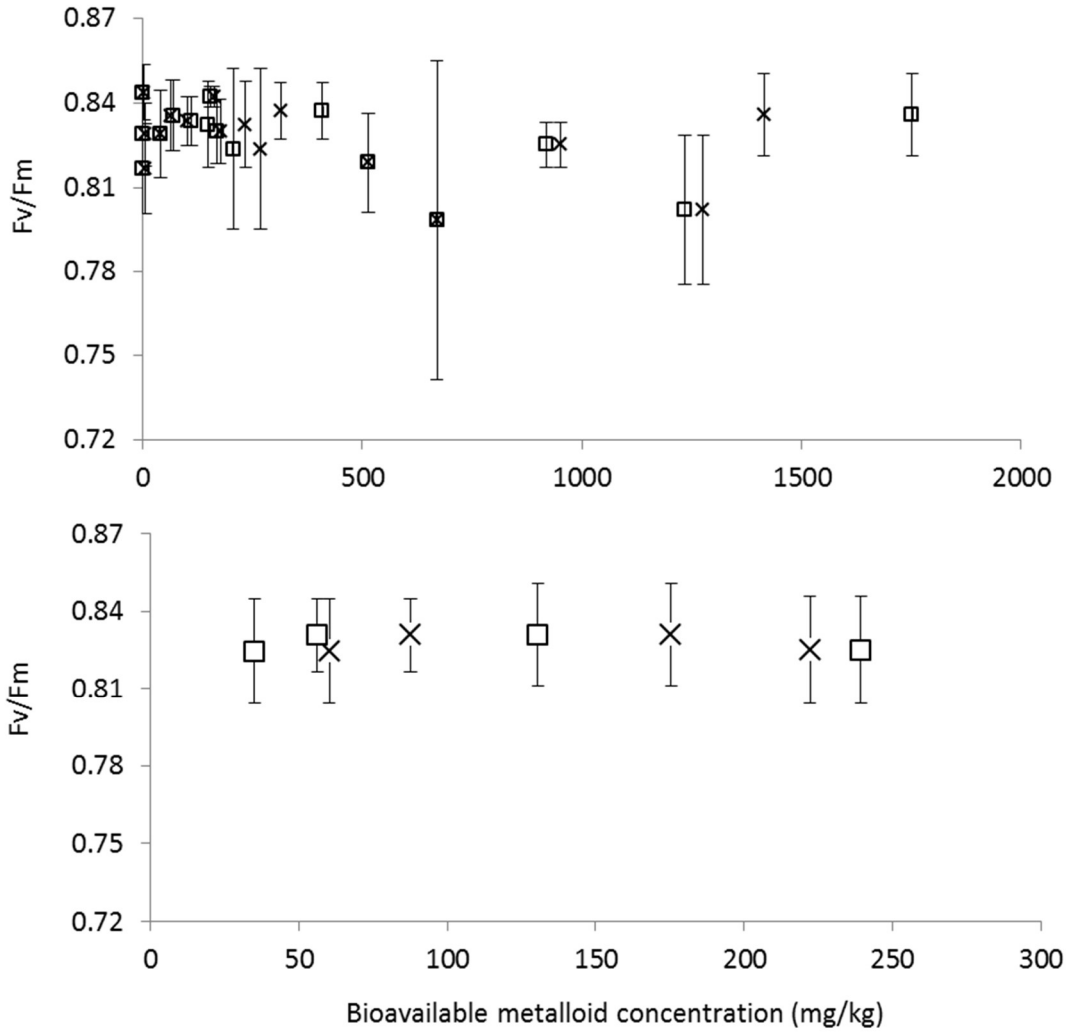

Figure A2.3. Photosynthetic efficiency (Fv/Fm) of *Ipomoea aquatica*, over increasing As (×) and Sb (□) concentrations (mg/kg) a). historically and b). recently co-contaminated soils

Appendix 3. Supplementary information for Chapter 4

Table A3.1. Wet mass, dry mass, and water content of water spinach in As $_{(Individual)}$, Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatments.

	Root			Shoot		
	Wet mass (g)	**Dry mass (g)**	**Moisture (%)**	**Wet mass (g)**	**Dry mass (g)**	**Moisture (%)**
Control	4.13	1.46	65	45.10	5.84	87
I1 $_{As}$	3.94	1.22	69	44.00	4.68	89
I2 $_{As}$	4.04	1.21	70	47.26	4.44	91
I3 $_{As}$	6.21	1.26	80	39.95	3.99	90
I4 $_{As}$	7.81	1.37	82	40.46	4.17	90
I5 $_{As}$	2.79	0.71	74	11.56	1.96	83
I6 $_{As}$	1.61	0.68	58	7.92	1.71	78
I1 $_{Sb}$	3.95	0.99	75	49.41	4.35	91
I2 $_{Sb}$	4.97	1.11	78	43.71	4.33	90
I3 $_{Sb}$	3.99	1.02	74	53.96	5.03	91
I4 $_{Sb}$	4.85	1.27	74	55.00	6.07	89
I5 $_{Sb}$	7.81	1.44	82	65.86	6.00	91
I6 $_{Sb}$	6.60	1.57	76	64.32	6.57	90
C1 $_{Sb + As}$	1.49	0.88	41	39.17	2.72	93
C2 $_{Sb + As}$	2.92	0.89	70	34.98	2.79	92
C3 $_{Sb + As}$	3.89	1.07	72	30.23	2.75	91
C4 $_{Sb + As}$	3.11	1.05	66	28.34	2.26	92
C5 $_{Sb + As}$	3.11	1.00	68	16.08	1.62	90
C6 $_{Sb + As}$	0.51	0.30	40	2.80	0.85	70

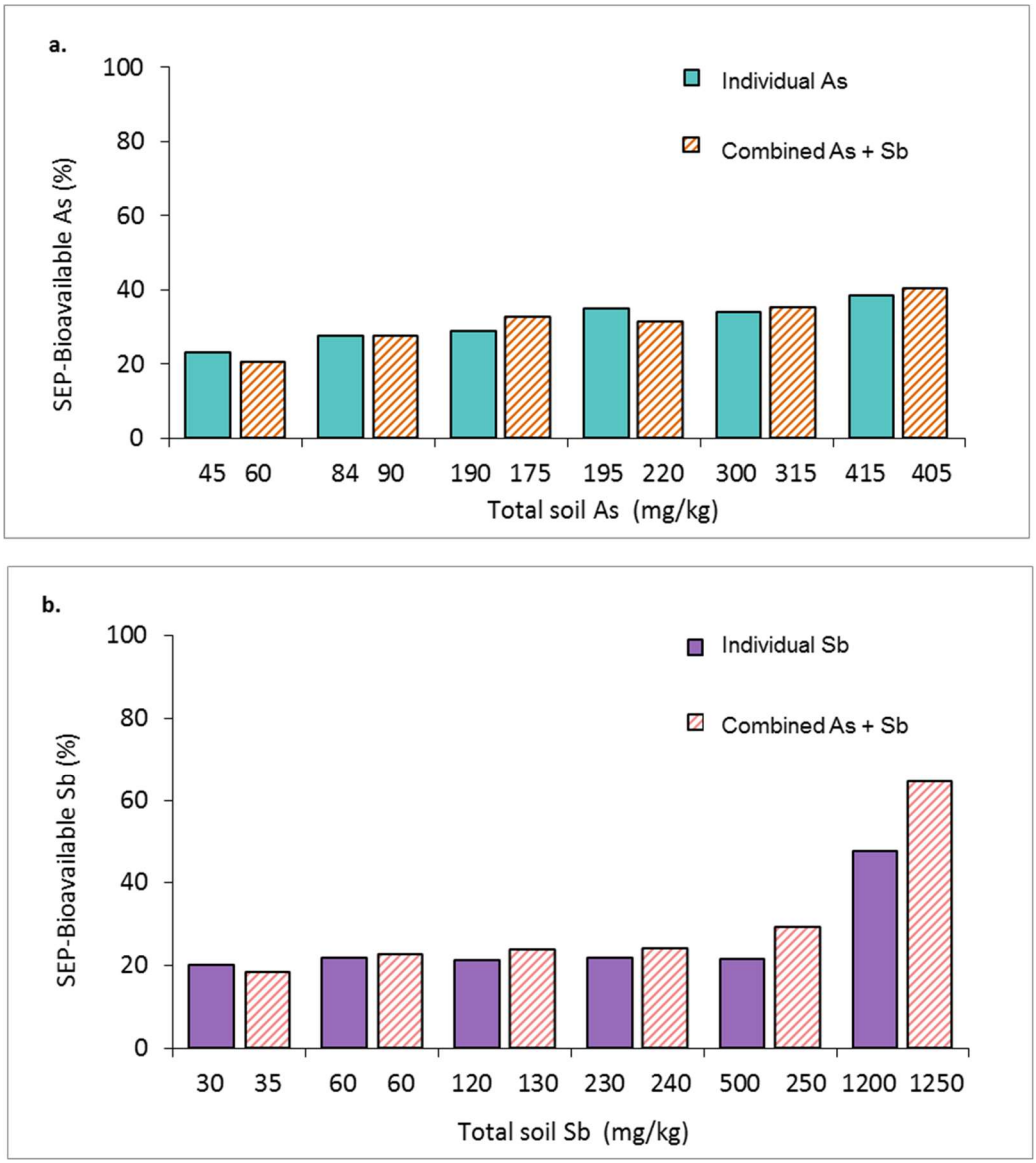

Figure A3.1. The SEP-bioavailable As and Sb fraction (percent of total soil metalloid concentration) for a). As $_{(Individual)}$ and As in As + Sb $_{(Combined)}$ treatments b). Sb $_{(Individual)}$ and Sb in As + Sb $_{(Combined)}$ treatments.

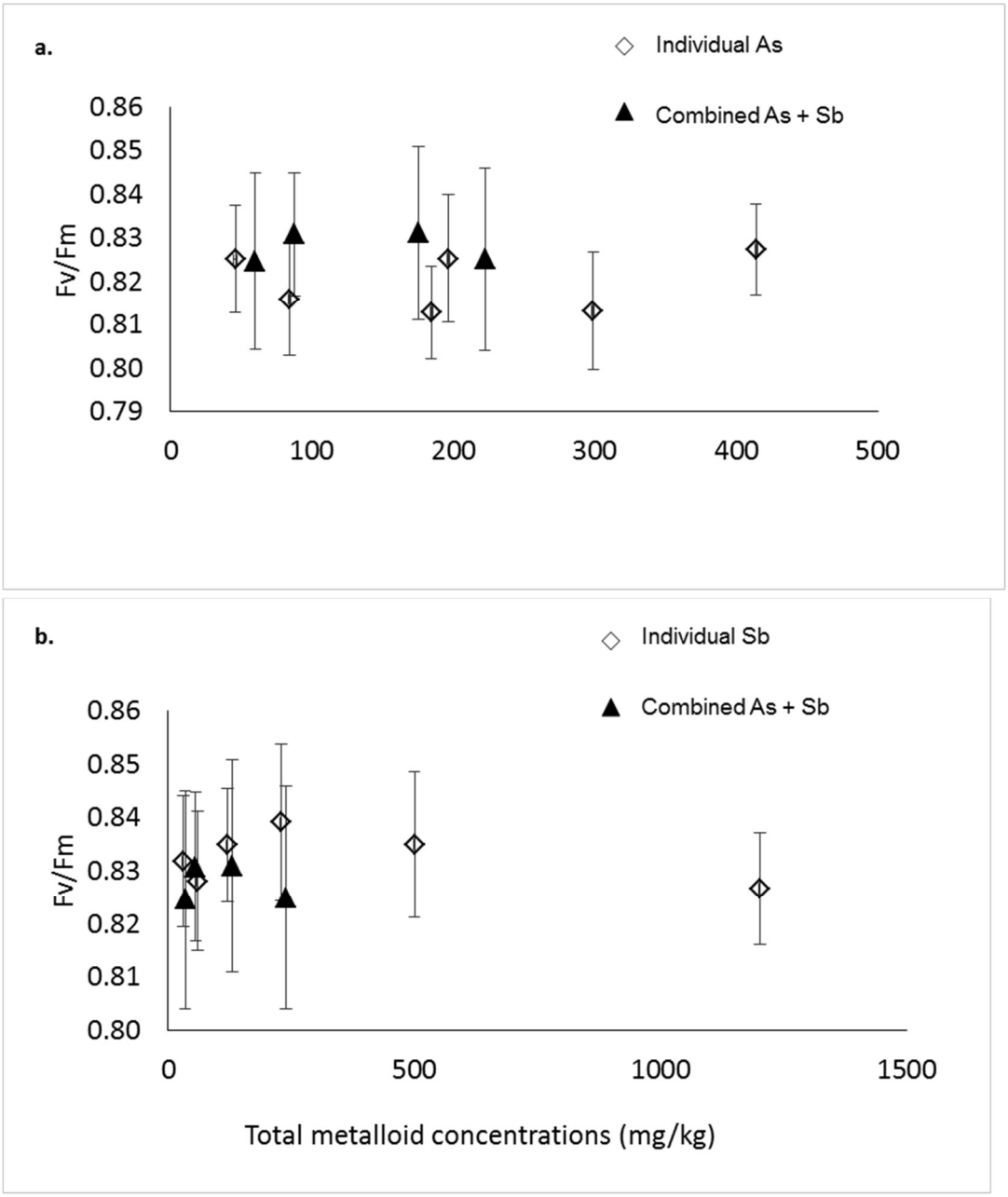

Figure A3.2. The maximum photosynthetic efficiency (Fv/Fm) in *Ipomoea aquatica* leaves across the concentration gradient for a). As and b). Sb when present individually and combined. C5 and C6 data missing as the plants had died (mean ± SD, $n \leq 3$).

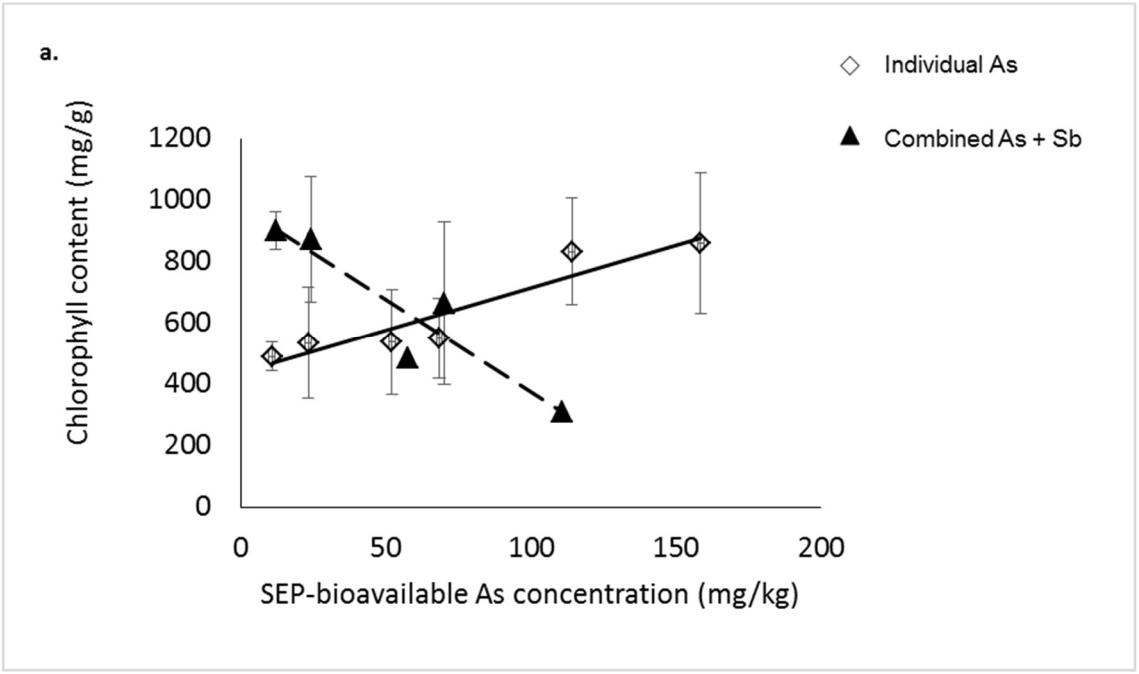

Figure A3.3. Chlorophyll *a* content in *Ipomoea aquatica* leaves across the concentration gradient for a). As b). Sb when present individually and combined (mean ± SD, n ≤ 3).

Appendix 4. Supplementary information for Chapter 5

Table A4.1. Soil physical and chemical properties (measured before amending with As and Sb and PO_4^{3-}). Soil properties were measured before amending with As and Sb and PO_4^{3-} . Soil pH and N were not measured after spiking and harvesting. All values are presented as the mean ± SD, n ≤ 3.

Soil characteristics	Measured value
Sand > 62.5 µm – 2 mm (%)	31 ± 2
Silt > 25-62.5 µm (%)	61 ± 2
Clay 3.9-25 µm (%)	7.9 ± 0.3
pH	5.43 ± 0.03
Soil moisture (%)	2.0-3.1
Total Organic Matter (%)	8.53 ± 0.07
Total Kjeldhal Nitrogen (mg N/kg)	543 ± 41

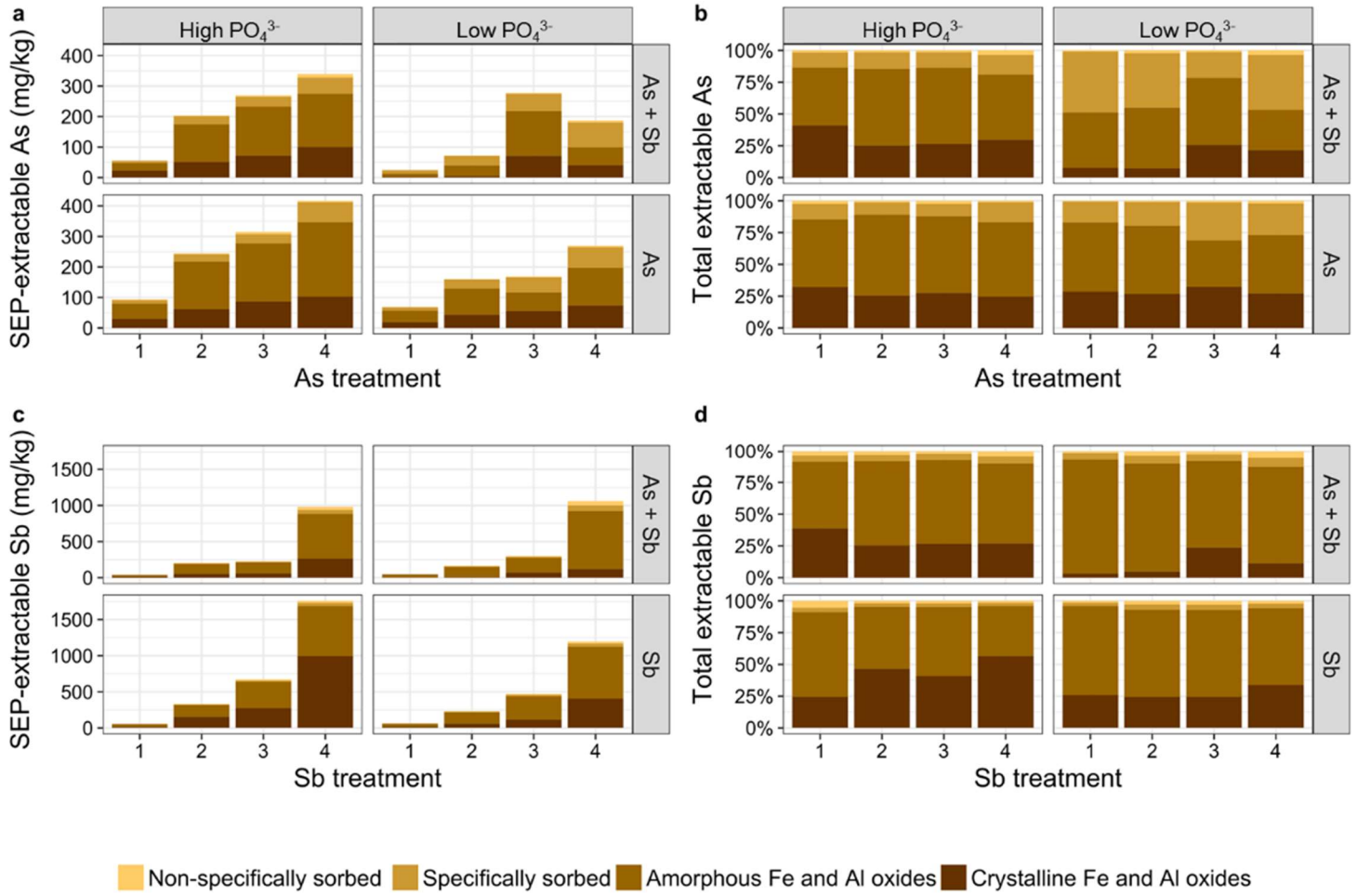

Figure A4.1. The association of As and Sb with different binding fractions in soils extracted by sequential extractions procedure (SEP) at low and high soil PO_4^{3-} concentrations when exposed to As contaminated soils (a and b) and Sb contaminated soils (c and d) ($n \leq 3$) as individual exposures and in combination. X-axes labels 1 to 4 represent the respective individual (I1 to I4) and combined (C1 to C4) treatments in Table 5.1.

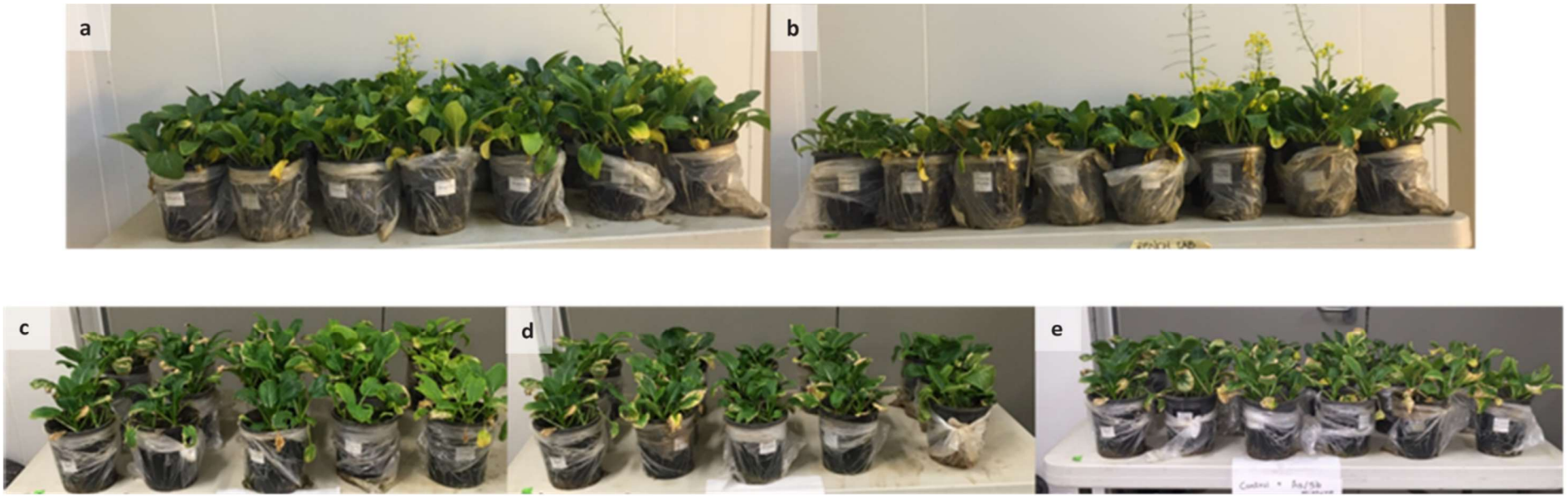

Figure A4.2. Growth of *Brassica chinensis* var. *parachinensis* across the concentration gradient (concentrations increase from left to right) in two bioassays, a, b). Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatment at low PO_4^{3-} concentrations, c, d, e). As $_{(Individual)}$, Sb $_{(Individual)}$ and As + Sb $_{(Combined)}$ treatment at high PO_4^{3-} concentrations, respectively.

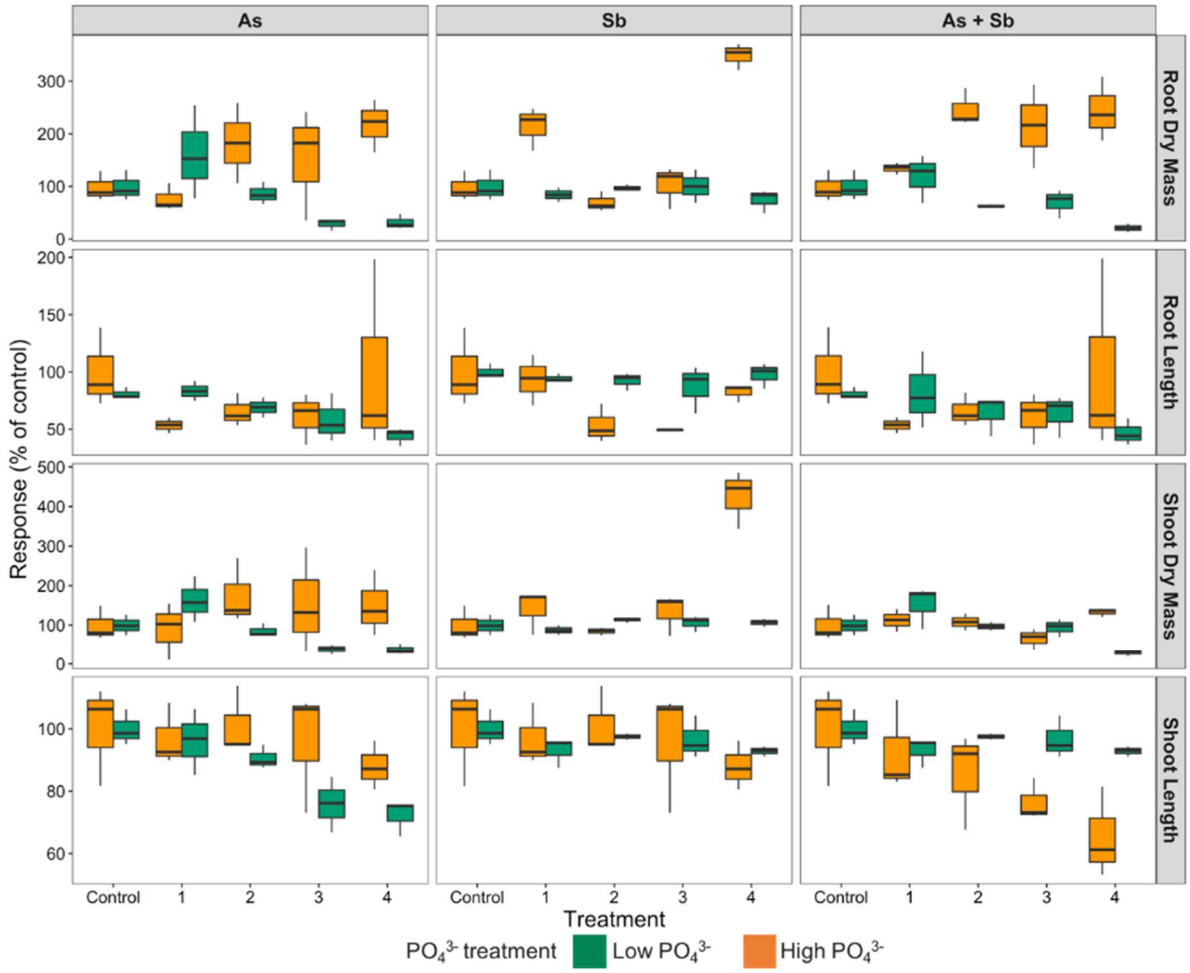

Figure A4.3. The change of root and shoot dry mass and length in As (Individual), Sb (Individual) and As + Sb (Combined) treatments at low and high PO_4^{3-} levels, mean ± SD, n ≤ 3.

Appendix 5. Supplementary information for Chapter 6

Table A5.1. The effect of As and Sb on root, shoot and grain dry mass, root and shoot lengths, number of tillers and panicle number in the As + Sb $_{(combined)}$ treatment. C4 plant data is not available (NA) due to plant death.

	Dry mass (g/pot)			Length (cm)		Number of tillers	Number of Panicles
	Root	Shoot	Grain	Root	Shoot		
Control (1)	2.23	13.61	10.87	29	58	21	23
Control (2)	14.19	25.04	16.32	21.5	66	31	38
C1 (1)	4.22	16.42	12.51	20.5	64	19	24
C1 (2)	2.74	17.89	12.19	20	58	23	28
C1 (3)	6.87	12.83	10.10	27	60	14	19
C2 (1)	1.99	9.83	4.79	22	60	11	11
C2 (2)	2.76	11.97	5.34	23	60	20	12
C2 (3)	2.36	10.57	10.9	28	62	11	23
C3 (1)	1.34	6.26	4.9	20	59	6	2
C3 (2)	2.62	9.52	0.38	23	55	11	10
C3 (3)	0.16	2.36	No grains	7	28	1	0
C4 (1)	NA	NA	NA	NA	NA	NA	NA
C4 (2)	NA	NA	NA	NA	NA	NA	NA
C4 (3)	NA	NA	NA	NA	NA	NA	NA

Table A5.2. The effect of As and Sb on root, shoot and grain dry mass, root and shoot lengths, number of tillers and panicle number in the As $_{(Individual)}$ and Sb $_{(Individual)}$ treatments. NA- not available as the plant died.

	Dry mass (g/pot)			Length (cm)		Number of tillers	Number of Panicles
	Root	**Shoot**	**Grain**	**Root**	**Shoot**		
As $_{(individual)}$							
I1 (1)	4.30	16.06	10.87	29	68	21	23
I1 (2)	2.28	12.74	10.30	26	65	18	16
I1 (3)	1.68	11.33	7.96	30	56	11	15
I2 (1)	2.76	12.63	8.40	27	63	14	18
I2 (2)	5.56	13.32	5.87	26	66	20	18
I2 (3)	3.27	14.85	10.84	25	72	19	21
I3 (1)	0.36	4.64		13	52	5	0
I3 (2)	1.20	5.90	1.09	30	57	8	5
I3 (3)	0.06	4.07		6	18	1	0
I4 (1)	NA	NA	NA	NA	NA	NA	NA
I4 (2)	NA	NA	NA	NA	NA	NA	NA
I4 (3)	0.79	9.15	6.55	24	57	12	8
Sb $_{(individual)}$							
I1 (1)	2.83	10.78	6.53	20.9	70	12	12
I1 (2)	6.72	22.33	10.41	21	56	25	30
I1 (3)	5.23	10.62	2.51	26	55	10	13
I2 (1)	4.68	17.17	11.29	20.4	58	24	27
I2 (2)	3.33	12.09	5.48	25.5	56	12	14
I2 (3)	9.71	20.08	13.26	21.5	61	27	32
I3 (1)	1.96	10.62	3.09	19	52	10	14
I3 (2)	5.61	16.53	10.18	23	60	18	24
I3 (3)	2.36	10.54	7.36	17	64	15	12
I4 (1)	0.68	6.68	0.80	21	58.5	9	3
I4 (2)	0.20	4.77	0	10	37	2	0
I4 (3)	4.88	19.55	9.95	19	58	20	29

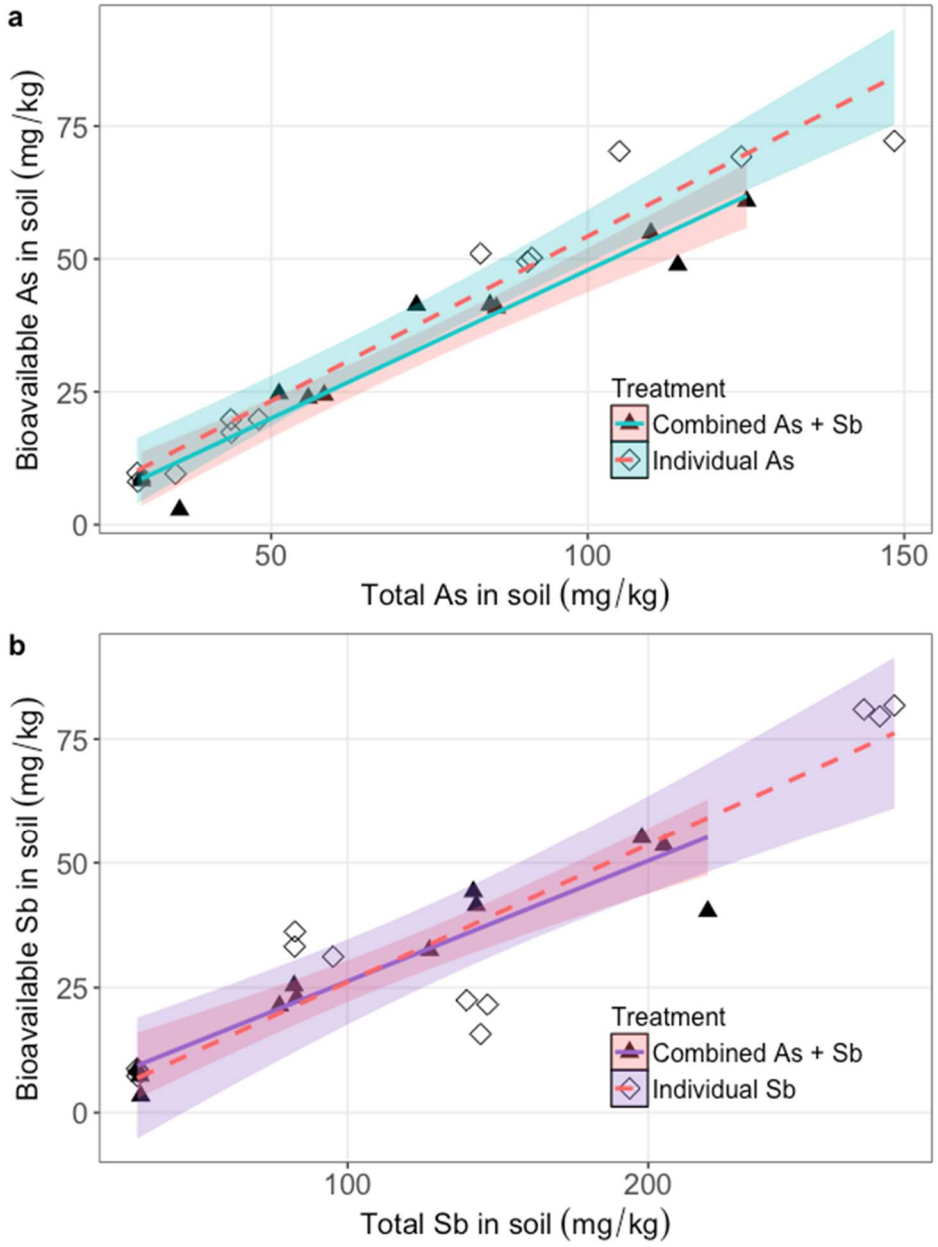

Figure A5.1. The effect of total metalloid concentrations in soils on SEP-bioavailable a). As and b). Sb fractions in soils.

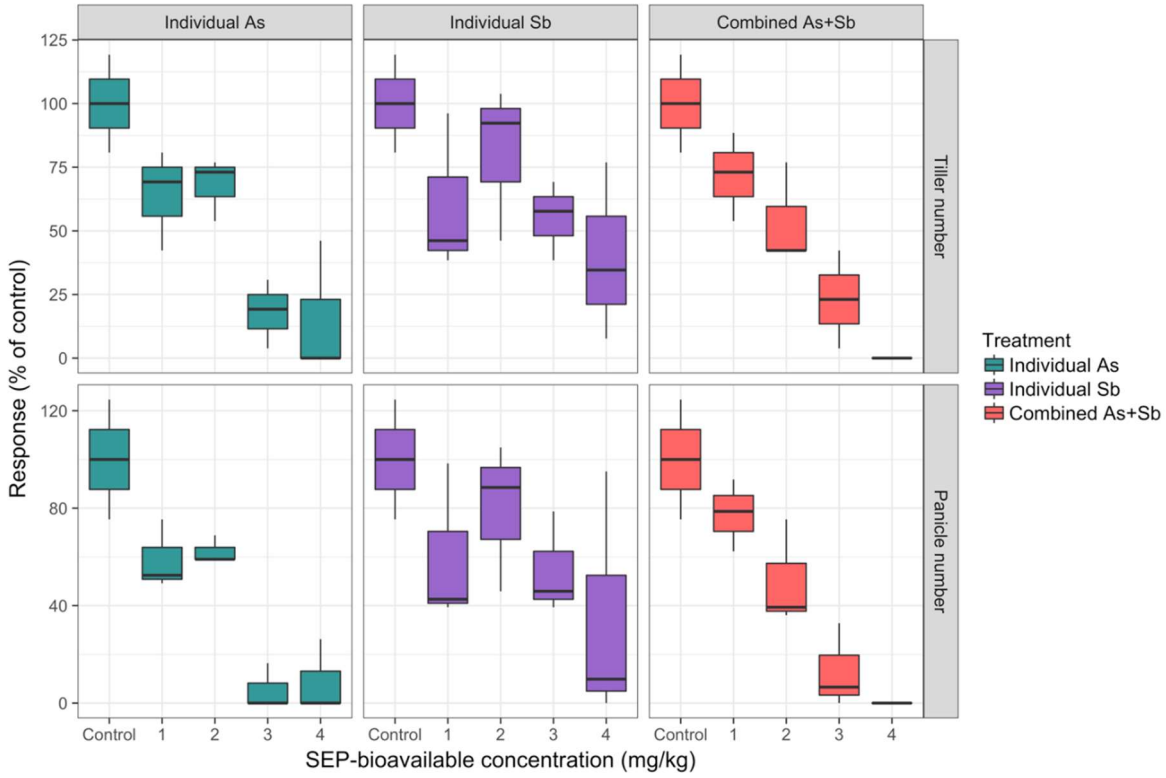

Figure A5.2. Individual and combined toxicity of As and Sb on growth of *Oryza sativa*. Individual As and Sb concentrations (labelled I1 – I4) and combined As + Sb concentrations are (labelled C1 – C4) are given in Table 6.2 (the boxes represent mean +/- 95% confidence limits).

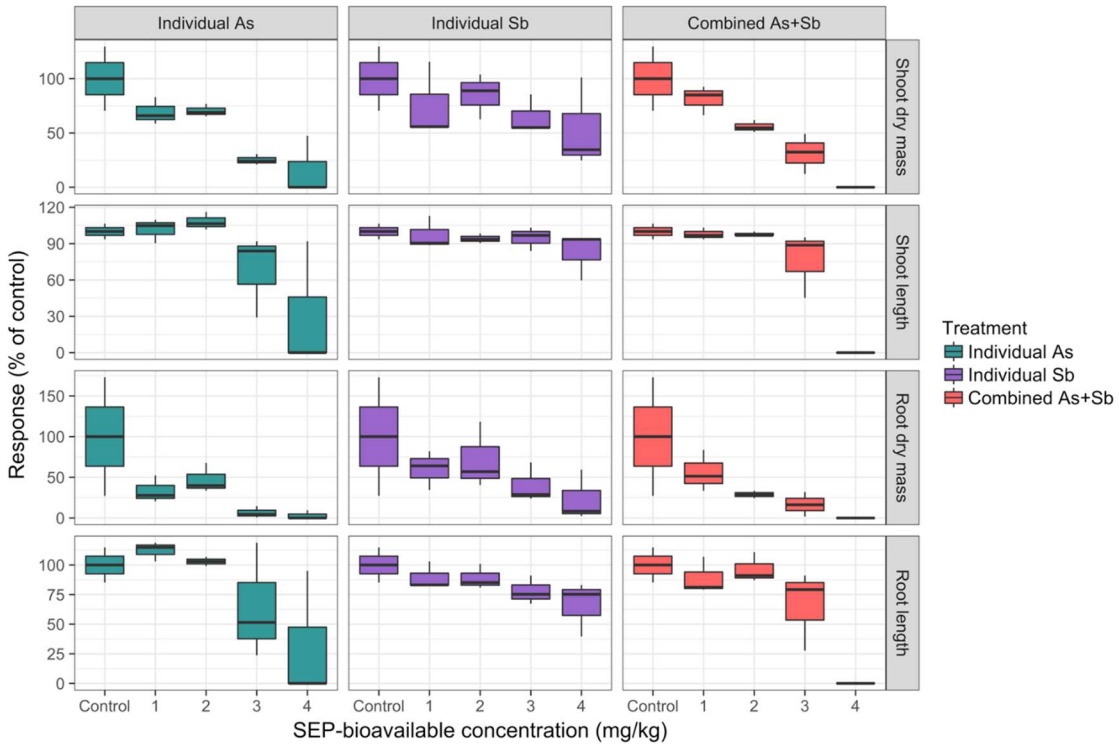

Figure A5.3. Individual and combined toxicity of As and Sb on growth of *Oryza sativa*. Individual As and Sb concentrations (labelled I1 – I4) and combined As + Sb concentrations are (labelled C1 – C4) are given in Table 6.2 (the boxes represent mean +/- 95% confidence limits).

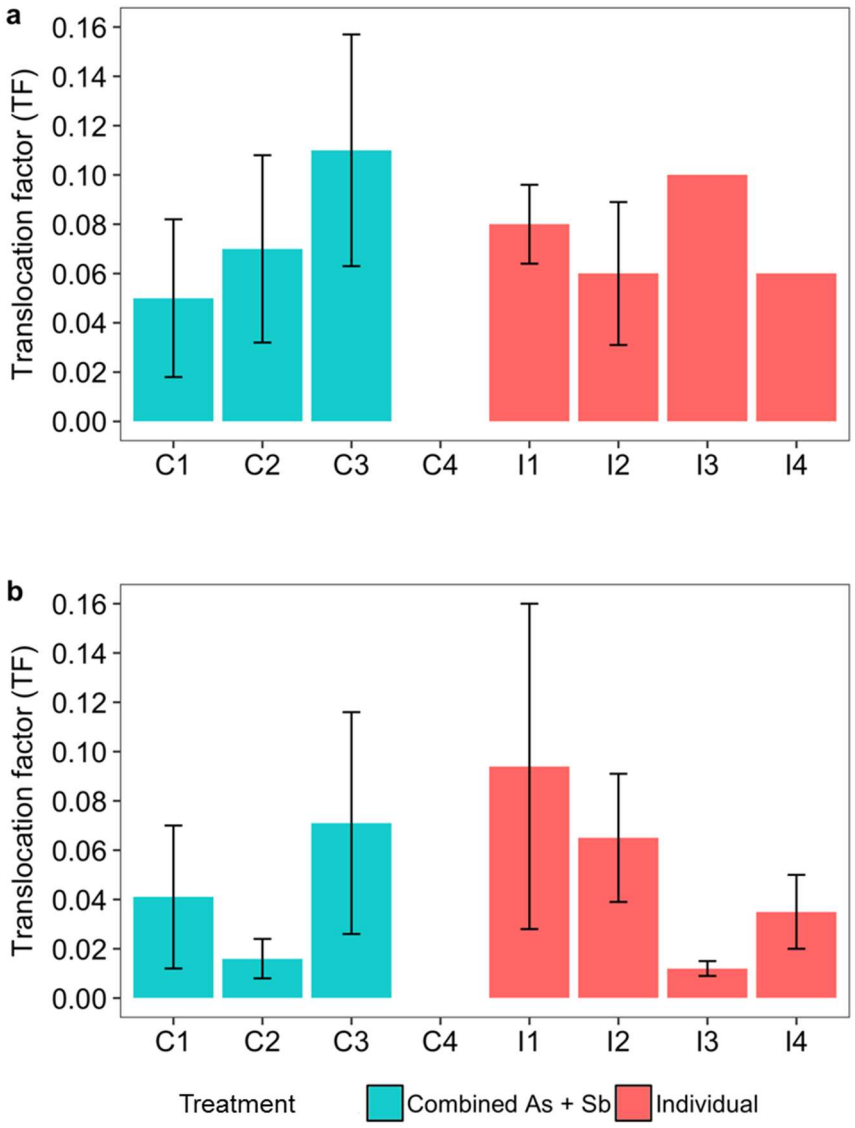

Figure A5.4. Translocation factor of a). As (C4 is not available and in I3 and I4 grains were available only in one plant as plants died) and b). Sb from *Oryza sativa* shoots to grains grown in individually and co-contaminated soils (mean ± SD, n ≤ 3).